*Vol. XXXVIII, No. 6 (December, 1957), was issued on 31.12.57.*

# THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXIX FEBRUARY, 1958 NO. 1

## ACQUIRED IMMUNOLOGICAL TOLERANCE TO A PROTEIN ANTIGEN IN CHICKENS

K. M. STEVENS, HELEN C. PIETRYK AND J. L. CIMINERA

*From the Department of Virology, Merck Sharp and Dohme Research Laboratories, West Point, Pa.*

Received for publication August 8, 1957

In 1953, Billingham, Brent and Medawar described the development of actively acquired immunological tolerance of mice and chickens to skin grafts following introduction of tissue from the future skin donor into the embryonic recipient. Since they used cellular systems, they felt that donor cells may have survived in the mature recipient. However, Hanan and Oyama (1954) and Dixon and Maurer (1955) demonstrated acquired tolerance to a purified protein following its injection into newborn rabbits.

The experiment here reported was an attempt to determine whether the injection of a protein antigen into the egg would specifically alter the subsequent antibody response to that antigen and whether the age of the embryo at the time of injection was critical.

The intravenous administration of concentrated solutions of one-half to one ml. volume is impractical in the chick embryo. However, it was found that bovine serum albumin, after intra-allantoic injection into 14-day eggs, was detectable in the blood of the chicks at 1 day of age (Stevens and Wildy, unpublished data). This demonstrated the feasibility of this route for the systemic distribution of albumin.

### MATERIALS AND METHODS

*Antigen preparations*

Soluble human serum albumin (HSA) was used as a 23 per cent solution stabilized with 0·02 M sodium caprylate and 0·02 M acetyl tryptophan (Biological Laboratories, Merck Sharp and Dohme, lot 3). To prepare 100 ml. of alum-precipitated HSA (AP–HSA), 8·7 ml. of HSA (2 g.) was diluted to 80 ml. in distilled water. Two g. of ALK $(SO_4)_2 . 12H_2O$ then were dissolved in the antigen solution, the pH adjusted to 6·5 with NaOH, merthiolate added so that the final concentration would be 1 : 10,000, and the solution brought to volume with water. Alum-precipitated bovine-$\gamma$-globulin (Armours Fraction II, lot 30008) (AP–BGG) was prepared in a similar way. The final pH was 7·0. Antigens were stored at 4°.

*Animal technics*

Fertile eggs were secured from white-Leghorn flocks and incubated at 36·5 ° and 50 per cent humidity. At ages varying from 8 to 18 days, groups of eggs were inoculated intra-allantoically (a.c.) with each egg receiving 115 mg. of soluble HSA. One group was inoculated into the yolk sac (y.s.). The eggs were sealed with Duco cement and allowed to hatch. At 6 weeks of age, when maximal responsiveness to protein antigens is reached in chickens (Wolfe and Dilks, 1948), five of the 10-day and five of the 18-day groups were bled, and the serum was tested for residual HSA. All chickens then were injected with 30 mg./kg. of AP–HSA intraperitoneally (I.P.) and bled from the heart 9 days later. Following a week's rest, all chickens were injected I.P. with 30 mg./kg. of AP–BGG as a control antigen and bled 9 days later.

*Antigen and antibody assays*

After clotting, the bloods were centrifuged at 5° and the sera removed and stored for 12 days at 4° (Wolfe, 1942). Following a few days in storage a precipitate that was removed by centrifugation formed. No more visible precipitate formed on further storage. One-ml. aliquots were placed in Kahn tubes and 0·5 ml. of 24 per cent NaCl added to each tube, producing a final concentration of 8 per cent NaCl (Goodman, Wolfe and Norton, 1951). For determination of residual HSA in the 10- and 18-day groups, the sera were layered with chicken antiserum containing 180 μg. of anti-HSA N/ml. in 8 per cent saline. For determination of antibody, 5 μg. aliquots of HSA-N or BGG-N were layered over the serum and incubated 20 min. at 37°. After inspection for precipitate, the tubes were shaken and centrifuged 5 min. at 2500 r.p.m. Further cycles of antigen addition, incubation and centrifugation were carried out until no precipitate was detectable at the interface. All tubes then were incubated 1 hr. at 37° and 4 days at 5°. The precipitates were centrifuged at 5° and washed twice with cold 8 per cent NaCl. Digestion was carried out with $H_2SO_4$ and selenium coated boiling stones (Selenized Hengar Granules, Hengar Co., Philadelphia, Pa.). Samples were distilled in a microstill and titrated with 0·0139 N HCL using methyl red containing methylene blue as indicator.

To determine that no non-specific precipitation occurred during the 4-day storage after addition of antigen, 10 normal 8-week-old chickens were bled and the sera treated as outlined. Five μg. of HSA–N were added to each serum and after the usual incubation and storage, precipitated N was determined. No precipitate was visible at any time and no nitrogen was detectable.

TABLE I.—*Variation and Proportionality of the Assay Method*

| Ml. serum | μg. Antibody N | | | |
|---|---|---|---|---|
| | HSA | | BGG | |
| | 1 | 2 | 1 | 2 |
| 1 | 30,32 | 239,— | 422,435 | 212,233 |
| 0·5 | 18,19 | 117,127 | 207,202 | 93,98 |
| 0·25 | 0,0 | 61,64 | 100,101 | 49,50 |

To determine the variation and proportionality of the assay method, two 10-week-old chickens were injected with 30 mg. of AP–HSA over a 2-week period and bled 1 week later. Two other chickens received AP–BGG. The sera from these birds were set up in duplicate using 1, 0·5, and 0·25 ml. of serum and brought to 1 ml. volume with 0·9 per cent NaCl. Adjustment of salt concentration and precipitation was then carried out as described. The results are shown in Table I. The mean variation between duplicates was 5 per cent and the mean variation referred to the amount of antibody present was 7 per cent. The values of zero listed under HSA 1 mean that the indicator did not change color when the sample was distilled. In our system, this required about 10 μg. of N so few values will be present between zero and 10. While this precipitation method is less exact than that of Heidelberger and Kendall, as described by Kabat and Mayer (1948), it is faster, requires less serum and is more exact than most methods that have been used in the study of acquired immunological tolerance.

TABLE II.—*Antibody Responses to HSA and BGG after Inoculation of HSA into the Egg at Different Ages*

$\mu$g. AbN/ml. serum

| Controls* | | 8-day y.s. | | 8-day a.c. | | 10-day a.c. | | 12-day a.c. | | 13-day a.c. | | 14-day a.c. | | 15-day a.c. | | 16-day a.c. | | 18-day a.c. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG | HSA | BGG |
| 14 | 280 | 14 | 92 | 14 | 188 | 0 | 80 | 9 | 114 | 0 | — | 0 | 0 | 0 | 68 | 14 | 140 | 0 | 175 |
| 26 | 188 | 18 | 110 | 30 | 87 | 24 | 82 | 10 | 159 | 0 | 187 | 0 | 255 | 0 | 28 | 24 | 34 | 0 | 72 |
| 29 | 460 | 24 | 53 | 43 | 488 | 28 | — | 14 | 265 | 19 | 49 | 0 | 18 | 9 | — | 48 | 95 | 0 | 25 |
| 30 | 53 | 34 | — | 53 | 247 | 41 | 380 | 14 | 40 | 20 | 308 | 0 | 160 | 19 | 104 | 48 | — | 18 | 180 |
| 38 | 139 | 43 | 24 | 82 | 570 | 43 | 302 | 14 | 179 | 24 | 212 | 0 | 326 | 25 | 170 | 58 | 316 | 23 | 146 |
| 40 | 174 | 47 | 207 | 82 | 96 | 66 | 123 | 18 | 233 | 27 | 17 | 0 | 316 | 29 | 190 | 59 | 217 | 25 | 137 |
| 44 | — | 68 | 105 | 82 | 164 | 79 | 289 | 23 | — | 29 | 210 | 0 | 326 | 39 | 52 | 87 | 279 | 31 | 190 |
| 105 | 86 | 86 | 255 | 84 | 43 | 81 | 165 | 23 | 69 | 29 | 200 | 0 | 174 | 45 | 229 | 121 | 204 | 37 | 121 |
| 111 | 154 | 112 | — | 275 | 537 | 94 | 79 | 23 | 208 | 39 | 165 | 14 | 75 | 49 | 24 | 130 | 295 | 76 | 150 |
| 150 | 183 | 129 | 227 | .. | .. | .. | .. | 28 | 401 | 39 | 58 | 28 | 267 | 58 | .. | 159 | 373 | 81 | 285 |
| 155 | 72 | 255 | 42 | .. | .. | .. | .. | 29 | 286 | 77 | 197 | 29 | — | 64 | 309 | .. | .. | 85 | 240 |
| 158 | 227 | 301 | 812 | .. | .. | .. | .. | 33 | 140 | 87 | 142 | 51 | 135 | 78 | 316 | .. | .. | 85 | 380 |
| 158 | 248 | .. | .. | .. | .. | .. | .. | 34 | 192 | 94 | 174 | 57 | 96 | 80 | 405 | .. | .. | 94 | 257 |
| 167 | 191 | .. | .. | .. | .. | .. | .. | 38 | 77 | 126 | 318 | 60 | 140 | 84 | 140 | .. | .. | 138 | 341 |
| 188 | 207 | .. | .. | .. | .. | .. | .. | 42 | 371 | 129 | 248 | 99 | 147 | 125 | 336 | .. | .. | 252 | 335 |
| 189 | — | .. | .... | .. | .. | .. | .. | 85 | 227 | 150 | 200 | 140 | 302 | 200 | 170 | .. | .. | .. | .. |
| 203 | 241 | .. | .. | .. | .. | .. | .. | 128 | 399 | 151 | 212 | .. | .. | .. | .. | .. | .. | .. | .. |
| 232 | 463 | .. | .. | .. | .. | .. | .. | 130 | 478 | 179 | 317 | .. | .. | .. | .. | .. | .. | .. | .. |
| 252 | 283 | .. | .. | .. | .. | .. | .. | 169 | 140 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. |
| 347 | 377 | .. | .. | .. | .. | .. | .. | 173 | 145 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. |
| 354 | 129 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. |

*Values in adjacent columns are for the same bird.
y.s. = yolk sac inoculation.
a.c. = allantoic cavity inoculation.

## RESULTS

No HSA was detectable in any of the 10 pre-injection sera from the 10- and 18-day groups 6 weeks after hatching.

In Table II are shown the individual antibody levels attained following AP-HSA and AP-BGG injections. Even in the control group, a 25-fold variation occurred in the anti-HSA values and a 9-fold variation in the anti-BGG values. Although the geometric mean response to BGG in the control group was almost double the response to HSA (Table III), there was no correlation between the response to the two antigens in individual birds.

TABLE III.—*Geometric means (μg. Ab N/ml. Serum) and Results of Rank Test*

| Age at inoculation | HSA n | HSA G.M. | HSA U.N.D. | HSA P | BGG n | BGG G.M. | BGG U.N.D. | BGG P |
|---|---|---|---|---|---|---|---|---|
| Controls | 21 | 101 | — | — | 19 | 190 | — | — |
| 8-day y.s. | 12 | 61 | −1·36 | 0·17 | 10 | 120 | −1·40 | 0·16 |
| 8-day a.c. | 9 | 61 | −1·38 | 0·17 | 9 | 195 | +0·25 | 0·80 |
| 10-day a.c. | 9 | 33 | −2·33 | 0·02 | 8 | 156 | −0·64 | 0·52 |
| 12-day a.c. | 20 | 34 | −3·35 | 0·001 | 19 | 182 | −0·09 | 0·93 |
| 13-day a.c. | 18 | 37 | −2·72 | 0·006 | 17 | 157 | −0·33 | 0·74 |
| 14-day a.c. (1) | 16 | 7 | −3·88 | 0·0001 | 15 | 114 | −0·66 | 0·51 |
| 14-day a.c. (2) | 37 | 24 | −4·10 | 0·00004 | — | — | — | — |
| 15-day a.c. | 16 | 30 | −2·59 | 0·01 | 14 | 141 | −0·66 | 0·51 |
| 16-day a.c. | 10 | 60 | −1·59 | 0·11 | 9 | 180 | +0·27 | 0·79 |
| 18-day a.c. | 15 | 26 | −2·70 | 0·007 | 15 | 171 | −0·35 | 0·73 |
| | | | | | Combined* | | −1·20 | 0·23 |

n = number of chickens.
G.M. = geometric mean.
U.N.D. = unit normal deviate. A negative value indicates a lower rank average for treated than controls; a positive value indicates a lower rank average for controls.
P = probability.
y.s. = yolk sac inoculation.
a.c. = allantoic cavity inoculation.

* In carrying out the combined tests it was assumed that the ratio of the sum of the individual estimates of unit normal deviates to the square root of the number of estimates was itself approximately distributed as a unit normal deviate:

$$\text{U.N.D.} = \frac{\Sigma\,(\text{U.N.D.})_1}{\sqrt{k}}$$

The effect of introduction of HSA into the egg is best shown in Table III where geometric mean responses for the different groups are given and the data are analyzed using the rank test of Kruskal and Wallis (1952). While complete tolerance was not found in any group, all groups except those injected at 8 and 16 days showed a significant decrease from the control response to HSA. There is no clear-cut correlation between the depression of the response to HSA and the age of inoculation, although the 14-day values were the lowest in both experiments. Since none of the responses to BGG differ significantly from the control, the *in ovo* injection of HSA appears to produce a specific inhibiting effect. Despite this, there is the suggestion that injection of HSA may cause a slight depression of response to BGG in that all of the unit normal deviate values for BGG are negative except for the 8-day and 16-day allantoic cavity inoculations, and these are the only two allantoic cavity inoculations which did not produce a significant depression to HSA.

Since the effect of sex and weight have not been commented on in other studies on antibody responses in chickens, these were analyzed. It was obvious on gross inspection that weight, in chickens of the same age, did not correlate with the antibody response.

The geometric mean antibody response to BGG was 143 for males and 211 for females. The difference is significant at the 1 per cent confidence level. However, when groups were analyzed separately by day of inoculation (Table IV),

TABLE IV.—*Results of Rank Test for Sex Difference**

| Age at inoculation | HSA | | | | BGG | | | |
|---|---|---|---|---|---|---|---|---|
| | No. M | No. F | U.N.D. | P | No. M | No. F | U.N.D. | P |
| Controls | 10 | 11 | +0·85 | 0·40 | 9 | 10 | +1·80 | 0·07 |
| 8-day y.s. | 5 | 7 | +1·21 | 0·23 | 4 | 6 | 0·00 | — |
| 8-day a.c. | 3 | 6 | +1·57 | 0·12 | 3 | 6 | +1·29 | 0·20 |
| 12-day a.c. | 14 | 6 | −1·16 | 0·25 | 14 | 5 | −0·05 | 0·96 |
| 13-day a.c. | 7 | 10 | +0·73 | 0·47 | 7 | 10 | +1·22 | 0·22 |
| 14-day a.c. (2) | 19 | 17 | +2·83 | 0·005 | — | — | — | — |
| 16-day a.c. | 4 | 6 | −0·21 | 0·83 | 3 | 6 | 0·00 | — |
| Combined† | .. | .. | +2·20 | 0·03 | .. | .. | +1·74 | 0·08 |

* A positive value of U.N.D. indicates a higher rank average for females; a negative value indicates a higher rank average for males.

† See note about combined tests under Table III.

a significantly higher value for females was found only in the second 14-day group of anti-HSA values, although the combined values for the groups indicate a higher response in females at about the 5 per cent confidence level. The sexes of the birds were not known for all ages, but it is doubtful if the sex ratio would alter the conclusions drawn from Table III except possibly for the 8-day a.c. group where two-thirds were females.

## DISCUSSION

The apparent absence of significant depression in the 8- and 16-day groups injected allantoically may be a matter of selection of birds. Both groups were small and, as noted earlier, these were the only groups showing a higher rank average for treated than controls in their response to BGG. If these groups responded better than controls to HSA as well, the observed difference from the controls would be less than expected so that the difference would not be statistically significant. Since the group injected into the yolk sac at 8 days had a much lower rank average than control birds given BGG, this argument cannot be used and this degree of depression is probably not significant.

This raises the question of entry of the antigen into the embryo. Hemmings (1956) has recently demonstrated that foreign protein, *e.g.*, bovine antibody, is very poorly absorbed from the chick yolk sac. Consistent with this was Buxton's (1954) inability to induce tolerance to *Salmonella pullorum* after injection into the yolk sac of 9-day eggs. Our results would indicate that HSA is poorly absorbed from the yolk sac but, like bovine serum albumin, is absorbed adequately from the allantoic cavity.

These results do not demonstrate that the time of introduction of the antigen is critical within the range of 8 to 18 days. The finding of the greatest depression

at 14 days is of some interest in that a profound fall in nucleic acid turnover has been reported at this time (Stevens, 1952).

In the field of acquired tolerance, several workers have secured positive results with chickens while others have had negative results. The results here reported clearly support the existence of the phenomenon in this species, irrespective of the mechanism involved. Our findings of only partial tolerance, coupled with the huge range of the normal response and the possible influence of sex, should be kept in mind when divergent results are considered.

On the negative side, Cohn (1957) studied the response to several protein and polysaccharide antigens given to 14-day chick embryos intravenously, and he found no depression. His dose of antigen was much smaller than was ours, and his birds received their second injection at 10–12 weeks of age. Green and Lorincz (1956) reported that the Krebs-2 ascites tumor, surviving in the chick embryo from the 11th to about the 18th day, did not confer tolerance on these birds as measured by their ability to form agglutinins to tumour cells on subsequent challenge. Their groups contained only 4 birds each, and the sex was not stated.

On the positive side, Buxton (1954) secured depression of the antibody response to *S. pullorum* when given to 15-day embryos but not when given to 20-day embryos. Tolerance to red cells has been demonstrated by Hasek, Hraba and Esslova (1956), Billingham, Brent and Medawar, (1955), Owen (1956), and Simonsen (1956). The last worker found a significant depression when chicks were injected at 17 to 19 days; none when injected at 10 to 13 days. Our results, using a protein antigen and a different route of administration, did not reveal any such critical age of injection.

## SUMMARY

One hundred and fifteen mg. of human serum albumin (HSA) were injected into the allantoic cavity or yolk sac (8-day only) of fertile eggs from 8 to 18 days old. At 6 weeks of age no circulating HSA was detectable in two groups tested. Birds then were given 30 mg./kg. of alum-precipitated HSA intraperitoneally (I.P.) and bled 9 days later. A week later, all chickens received 30 mg./kg. of alum-precipitated bovine-$\gamma$-globulin (AP-BGG) I.P. as a control antigen. Antibody N was determined by a quantitative precipitin technic.

The geometric mean values of anti-HSA for the combined experimental groups was 37 per cent of the control. There was large individual variation in antibody response and the data therefore were analyzed by a non-parametric rank procedure. Eggs injected at 10, 12, 13, 14, 15 and 18 days showed a significant depression of anti-HSA response while those injected at 8 and 16 days did not. However, other considerations suggest that depression probably occurred in those inoculated allantoically at 8 and 16 days as well. No significant depression occurred in the group in which antigen was inoculated into the yolk sac at 8 days.

While lowest values were secured from chickens first inoculated as 14-day-old eggs, no consistent pattern relating age of inoculation to subsequent antibody response emerged.

There were no significant differences between the controls and any of the experimental groups in their responses to AP-BGG.

Females tended to give somewhat higher responses to both antigens than did males.

There was no correlation between the response of an individual control bird to AP-HSA and the same bird's response to AP-BGG.

In chickens of the same age, there was no correlation between body weight and antibody response.

## REFERENCES


BILLINGHAM, R. E., BRENT, L. AND MEDAWAR, P. B.—(1953) *Nature*, **172**, 603.—(1955) *Experientia*, **11**, 444.

BUXTON, A.—(1954) *J. gen. Microbiol.*, **10**, 398.

COHN, M.—(1957) *Ann. N.Y. Acad. Sci.*, **64**, 859.

DIXON, F. J. AND MAURER, P. H.—(1955) *J. exp. Med.*, **101**, 245.

GOODMAN, M., WOLFE, H. R. AND NORTON, S.—(1951) *J. Immunol.*, **66**, 225.

GREEN, H. AND LORINCZ, A. L.—(1956) *Nature*, **178**, 146.

HANAN, R. AND OYAMA, J.—(1954) *J. Immunol.* **73**, 49.

HASEK, M., HRABA, T. AND ESSLOVA, M.—(1956) *Folia Biologica*, **2**, 54.

HEMMINGS, W. A.—(1956) *Proc. roy. Soc.* (B), **145**, 186.

KABAT, E. A. AND MAYER, M. M.—(1948) ' Experimental Immunochemistry '. Springfield (Charles C. Thomas), p. 59.

KRUSKAL, W. H. AND WALLIS, W. A.—(1952) *J. Amer. statist. Ass.*, **47**, 583.

OWEN, R. D.—(1956) *Proc. roy. Soc.* (B), **146**, 8.

SIMONSEN, M.—(1956) *Acta path. microbiol. scand.*, **39**, 21.

STEVENS, K. M.—(1952) *Cancer Res.*, **12**, 62.

WOLFE, H. R.—(1942) *J. Immunol.*, **44**, 135.

*Idem* AND DILKS, E.—(1948) *Ibid.*, **58**, 245.

# THE SPECIFICITY OF ACQUIRED IMMUNOLOGICAL TOLERANCE TO AZO PROTEINS

B. CINADER AND J. H. PEARCE*

*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

Received for publication September 6, 1957

EARLIER work on the specificity of acquired immunological tolerance to human albumin (H.A.) has shown that a majority of rabbits unable to form antibody to H.A. are also unable to form antibody to azo human albumin (D.H.A.). Those rabbits which did respond to D.H.A. formed antibody primarily adapted to the modified portions of the protein, but nevertheless capable of reacting with H.A., though with a remarkably low avidity (Cinader and Dubert, 1955).

Serum proteins of mammals cross-react with one another and are thus structurally similar. Acquired immunological tolerance may consequently be more readily induced by foreign serum proteins than by antigens of a structure which is radically different from that of the naturally circulating proteins of the injected new-born animal. For this reason alone the possibility of acquired immunological tolerance to D.H.A. deserved attention ; the first part of this paper is concerned with the effect of injections of D.H.A. at birth on the subsequent response to D.H.A.

The second part of this investigation deals with the effect of the injection of one azo protein on the subsequent response to another azo protein, the diazo groups being the same. We were thus examining the cross-reaction of acquired immunological tolerance to modified proteins. This problem arose not only in the context of studies on acquired tolerance but also in that of antibodies against enzymes (Cinader, 1957*a*). It was therefore of some interest to examine whether the injection of azo-rabbit serum into new-born rabbits would prevent these rabbits from responding during adult life to a heterologous antigen coupled with the same diazonium salt and if the rabbits responded, whether the resulting antibody would be adapted exclusively to the protein moiety of the antigen or to both the protein and the synthetic group.

Several reports have stressed the absence of haem specificity in antibodies to haemoglobin and catalase. This could be ascribed to acquired immunological tolerance to haem groups on the assumption that the same mechanism is responsible for acquired immunological tolerance and for the prevention or limitation of the synthesis of auto-antibodies. It is clear that a chemically modified protein will not necessarily represent an adequate model system. To choose an adequate model, information must be accumulated on the nature of cross reactivity in acquired immunological tolerance. In this context the size of the chemical grouping and nature and stability of its link to the protein may be the decisive feature. From neither of these tentative points of view does an azo protein resemble a haemoglobin. The following experiment can thus only represent a first explora-

* Present address : Microbiological Research Establishment, Porton, Wiltshire.

tion in the direction indicated. A specific example has been quoted in the haem proteins to illustrate reasons for the continued examination of a wider problem, that of the nature of cross reactivity in immunological tolerance.

The following systems of proteins coupled with the diazonium salt of *p*-amino benzene sulphonic acid were investigated :

1. Injection immediately after birth : azo human albumin (D.H.A.) ; injection in adult life : azo ribonuclease (D.Rn (18)).
2. Injection immediately after birth : azo rabbit serum (D.R.S.) ; injection in adult life : azo human albumin (D.H.A.).
3. Injection immediately after birth : azo ribonuclease (D.Rn) ; injection in adult life : azo human albumin (D.H.A.).

The antibodies in rabbits from the above three groups were examined for their protein and for their azo specificity (including such new specificity as may be imposed on the protein structure itself in consequence of diazotisation) ; only in the first of the three groups was the response to the antigen injected at birth investigated by the response in adult life to the same antigen. In group 3 this was frustrated by the poor antigenicity of D.Rn and Rn in adult white Himalayan rabbits ; in group 2 it was not attempted.

## MATERIALS AND METHODS

*Protein antigens*

The immunological properties of the native proteins used in this investigation have been described in earlier publications (human albumin (H.A.) : Cinader and Dubert, 1955 ; bovine ribonuclease (Rn) : Cinader, Rondle and Pearce, 1955 ; Cinader and Pearce, 1956). The preparation and properties of the azo human albumin (D.H.A.) used in the following experiments has been reported by Cinader and Dubert (1955, 1956).

Ribonuclease and rabbit sera were coupled with diazonium salt in an ice water bath. The diazonium salt of sulphanilic acid, cooled to 0°, was added drop by drop to the protein solutions and the pH was kept between 8·0–9·0 by the addition of 1 N NaOH.

Sulphonic acid containing molecules labelled with $S^{35}$ was used to measure the number of chemical groups introduced into Rn. The radioactivity in thoroughly dialysed preparations of the chemically modified proteins was compared with that of the solution of sulphonic acid used in the preparation of the diazonium salt.

*Azo ribonuclease* (D.Rn).—D.Rn was prepared from crystallised bovine ribonuclease (Armour). The protein was coupled with the diazonium compound of benzene-*p*-sulphonic acid.

Two products were prepared which differed in the degree of coupling.

1. *D.Rn* (7).—The concentration of the diazonium salt was adjusted so that 0·16 mg. sulphanilic acid were added for each mg. of ribonuclease. The ratio between the S contained in the azo groups and the protein nitrogen was 0·107.

2. *D.Rn* (18).—The concentration of the diazonium salt was adjusted so that 0·48 mg. sulphanilic acid were added for each mg. of ribonuclease. The ratio between the S contained in the azo groups and the protein nitrogen was 0·282.

The modification of these two preparations of azo ribonuclease can be judged from their reaction with ribonuclease antibody. While D.Rn (7) precipitates nearly as much of this antibody as does Rn, D.Rn (18) precipitates only a small fraction of ribonuclease antibody (Fig. 1).

*Azo-rabbit serum* (*D.R.S.*).—The concentration of the diazonium salt was adjusted so that 0·50 mg. sulphanilic acid were added per mg. of protein.

*Reagents*

Reagents used in the estimation of nitrogen by Microkjeldahl were M.A.R. grade. Other reagents were A.R. grade and were dissolved in glass distilled water.

*Polyvidone.*—Polyvinyl pyrrolidone (free of disinfectants) was obtained from May & Baker Ltd. as a 25 per cent solution.

*Polyvidone saline.*—Polyvinyl pyrrolidone (0·35 per cent) in 0·15 M-NaCl.

*Sulphanilic acid* ($S^{35}$).—Radiochemical purity was examined by isotope dilution analysis and was found to be 99 per cent compared with laboratory reagent sulphanilic acid. Paper chromatography in a mixture of ethanol, ammonia and water (80 : 4 : 16) showed one spot of $R_F = 0·5$ on autoradiography and did not show a spot corresponding to sulphate ion ($R_F = 0·1$).

*Immunological methods*

*Strain of rabbits.*—The rabbits used in these experiments were white Himalayan rabbits obtained from the National Institute for Medical Research, except 87A, C, D, E, H, 88E,

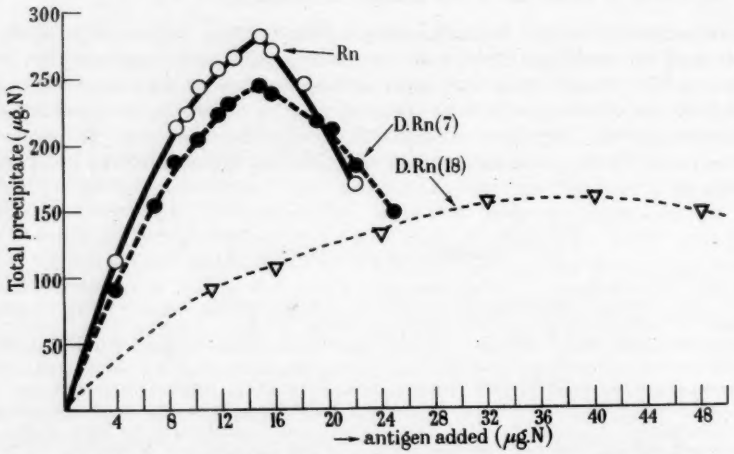



FIG. 1.—Precipitation of Rn, D.Rn (7) and D.Rn (18) by ribonuclease antiserum (Pool 3).

▽---▽ quantitative precipitin curve for D.Rn (18) and Rn antiserum.

○——○ quantitative precipitin curve for Rn and Rn antiserum.

●- - -● quantitative precipitin curve for D.Rn (7) and Rn antiserum.

110, 111, 112 and 114 which were wild type rabbits and 63 M, A, C, E which were red New Zealand rabbits.

*Designation of rabbits.*—Individual rabbits were distinguished by a number shared by all the individuals of the same litter and a letter (A to H); the mothers were described by the number of their litter and the letter M.

*Injections of new-born rabbits.*—New-born rabbits were injected intraperitoneally with protein solutions dissolved in saline (0·15 M-NaCl); the first injection was given within 12 hr. of birth.

*Injections of adult rabbits.*—Injections were made into the marginal vein of the ear of protein dissolved in saline. A course of injections consisted usually of three injections administered at intervals of 48 hr. Rabbits were bled 11 days after the first course and 5 (sometimes also 11) days after the last injection of the second and subsequent courses.

*Agglutination* (see Boyden, 1951; Borduas and Grabar, 1953).—Sheep erythrocytes were washed three times with saline, suspended in phosphate-saline buffer (pH 7·2) to give a 2·5 per cent suspension and were then mixed with an equal volume of tannic acid (1/20,000) in saline; after 10 min. the tanned erythrocytes were centrifuged, washed with phosphate buffer at pH 7·2 and suspended in saline. The concentration of this suspension of tanned cells was so adjusted that when 0·5 ml. was added to 7·5 ml. of distilled water and treated

with coal gas for 2 min., the optical density of the resulting solution was 0·3 (EEL Colorimeter, Ilford filter No. 624) in a tube 1·5 cm. in diameter.

To 10 ml. of this suspension were added 40 ml. phosphate buffer pH 6·4 containing 2·5 mg. of antigen. After 15 min. the tanned and sensitised erythrocytes were centrifuged and washed three times with polyvidone saline solution.

The concentration of these tanned and sensitised cells was adjusted as before.

Sera were heated in stoppered tubes at 56° for 60 min. Natural sheep antibodies were absorbed for 1 hr. with 0·1 ml. of washed centrifuged sheep erythrocytes per ml. of serum and the erythrocytes were then removed by centrifuging. Sera were diluted in polyvidone-saline, and to each of the serum dilutions was added one drop of the suspension of tanned and sensitised sheep erythrocytes from a pipette calibrated to deliver 0·05 ml. per drop. We were unable to obtain any results with cells which we attempted to sensitise with D.Rn (18), we have therefore assayed sera obtained after immunisation with either D.Rn (7) or D.Rn (18) with tanned erythrocytes sensitised with D.Rn (7).

*Examination of the specificity of antibodies by agglutination inhibition.*—This method has been applied in earlier studies of acquired tolerance (Cinader and Dubert, 1955) and in the comparison of ribonuclease A and B (Cinader and Pearce, 1956). Tanned sheep erythrocytes were sensitised with azo proteins and were agglutinated with antibody formed in response to these azo proteins. The agglutination titre was reduced if the native protein was allowed to react with the immune serum before tanned and sensitised cells were added.

Different quantities of inhibitors dissolved in saline, 0·15 ml. of immune serum, and a quantity of polyvidone to give a final concentration of 0·35 per cent, were made up with saline to give a final volume of 1·5 ml. The mixtures were incubated for 2 hr. at 37°, the kept at $2 \pm 1°$ overnight and were finally centrifuged. The agglutination titres of the immune sera were subsequently examined and compared with those of sera to which antigen had not been added. The azo proteins used in these experiments were D.H.A. and D.Rn and the antigens used for inhibition were principally H.A. and Rn.

*Nitrogen assays of antigen-antibody precipitates.*—Varying amounts of antigen were added to constant quantities of antibody and were immediately mixed to reduce the Danysz effect (Danysz, 1902; see also Cinader, 1957*b*). Mixtures were adjusted to constant volume with phosphate saline buffer (pH 7·5). The mixtures were kept at 37° for 2 hr. and then at 2° for 6 days. The precipitates were centrifuged at 4°, washed twice with 2 ml. of ice cold phosphate saline buffer and then assayed for nitrogen by the micro-Kjeldahl method.

*The determination of heterologous antigen in the blood stream of rabbits.*—Rabbits were given 3 intravenous injections of 10 mg. of H.A. at intervals of 48 hr. and were bled on the fifth, eleventh and eighteenth day after the last injection. The blood was allowed to clot and serum was separated.

Sera from rabbits immunised by repeated courses of intravenous injections with H.A. were pooled; two such pools were used. The nitrogen precipitated by varying known quantities of H.A. from a constant volume of each of these pooled sera was determined, and a calibration curve was drawn by plotting antigen N added against total N precipitated.

Rabbit sera to be assayed for human albumin were added to the same quantities of pooled H.A. antisera; the precipitated nitrogen was measured and compared with the nitrogen precipitated by known quantities of H.A. Samples of unknown antigen content were usually determined in the region of excess antibody.

The experimental procedure was adjusted so that the conditions in which the calibration curve was determined corresponded closely to those prevailing in the determination of the quantity of H.A. in the serum of injected rabbits. To this end all mixtures of antigen and antibody were adjusted by the addition of normal rabbit serum to contain finally the same amounts of rabbit serum.

All mixtures of antigen and antibody were incubated for 2 hr. at 37° and then kept for 6 days at 2°. Precipitates were isolated by centrifugation at 4°, washed twice with phosphate saline and assayed for nitrogen.

The half life of the antigen was calculated from the equation

$$\text{half life} = \frac{t}{\log n_1 - \log n_2} \times 0{\cdot}301$$

where $n_1$ and $n_2$ was the amount of human albumin found in 1 ml. of rabbit serum on the fifth and eleventh day respectively and $t$ was the time in days (6) between the two determinations.

## RESULTS

### *The response to injections with H.A.*

It has been shown (Cinader and Dubert, 1955) that 20 injections of 1 mg. of H.A. given to new-born rabbits caused immunological tolerance. The effect of smaller numbers of injections was examined. A single injection of 5 mg. of H.A. did not seem to affect the immunological response to the same antigen given in later life in four out of five rabbits (Table I).

Rabbits (88E, 266A) injected four times with 10 mg. H.A. during the first 3 days of their life received during adult life three courses of immunisation with H.A., each consisting of three injections at intervals of 48 hr. During the first course 15 mg. and during each of the two subsequent courses 30 mg. of H.A. were administered. The agglutination titre to H.A. was measured 5 and 11 days after the last injection of each course of immunisation and was found to be less than 1/5 in every instance.

TABLE I.—*The Response to H.A. During Adult Life of Rabbits Injected Intraperitoneally with 5 mg. H.A. Immediately After Birth and of Controls*

(*a*) Intraperitoneal injections immediately after birth.

| | | |
|---|---|---|
| Litter | 87† | 63† |
| Individuals injected | E, H | E*, G, H* |
| Quantity of H.A. injected (mg.) | 1×5 | 1×5 |

(*b*) Dose injected into adult rabbits.

| | | |
|---|---|---|
| Total mg. H.A. injected in each course (at intervals of 48 hr.) | 3×5 . 3×10 . 1×20 . 3×10 | 3×5 . 3×10 . 3×10 . 3×10 |

(*c*) Agglutination titre of tanned erythrocytes sensitised with H.A.

| Bleedings‡: days after birth | | 80 | 100 | 118 | 339 | 71 | 77 | 97 | 124 | 262 |
|---|---|---|---|---|---|---|---|---|---|---|
| Rabbits | A | <5 | 200 | 1,000 | 20,000 | § | § | § | § | § |
| | C | <5 | <5 | <5 | 1,000 | 500 | 500 | 500 | 500 | 20,000 |
| | D | 2,000 | § | § | § | § | § | § | § | § |
| | E | **<5** | **200** | **2,000** | **10,000** | **100** | **<5** | § | § | § |
| | G | § | § | § | § | **<5** | **<5** | **<5** | **<5** | **1,000** |
| | H | **200** | § | § | § | **<5** | **<5** | **<5** | **<5** | **<5** |
| | M | § | § | § | § | <5 | <5 | <5 | <5 | 5,000 |

* These rabbits received an injection of 1 ml. normal rabbit serum together with the injection of H.A.

† The animals in both these litters were severely affected by coccidiosis during the 6th and 7th weeks of their life and the poor and slow antibody response may be due to this fact.

‡ Bleedings: 11 days after the last injection of the first course, and 5 days after the last injection of subsequent courses.

§ Died

Titres in heavy type are measured on sera from animals injected at birth.

### *Response during adult life to injections with D.H.A. of rabbits injected with D.H.A. immediately after birth*

Details of the injections given to new-born rabbits and the response of adult animals to injection of D.H.A. are shown in Table II. A single injection of 5 mg. D.H.A. within the first few hours after birth did not affect the subsequent immuno-

logical response of the rabbit. Twenty post-natal injections of 1 mg. each or of 4 mg. each or four post-natal injections of 10 mg. each depressed or inhibited completely the subsequent response to D.H.A. during adult life. The observed effect differed from that found where H.A. was injected at birth and subsequently during the adult life of the rabbit. While the injection with H.A. suppressed a later response to this antigen either completely or not at all, the injection with D.H.A. at birth allowed in some animals a low residual response to injections with D.H.A. when the animal was injected after the second month of its life. This response which could only be demonstrated by agglutination of tanned and sensitised erythrocytes, was very weak, and differed from normal immunological responses by a decrease of antibody titre in response to successive courses of injections. The extent of this response and the degree of its decline after successive injections was variable even within a group of animals from the same litter injected in the same manner. This was most noticeable when the response of rabbits 146A, 146B and 146C was compared (Table II).

It seemed possible to attribute these differences observed in parallel experiments with H.A. and D.H.A. to the different rate of elimination of these two antigens (Cinader and Dubert, 1956). H.A. is relatively slowly eliminated from the blood stream of normal rabbits and might therefore mask the release of small quantities of antibody. D.H.A. is very rapidly eliminated and the presence of small quantities of antibody in the circulation might, therefore, be more readily demonstrable. To test this possibility the elimination of H.A. from the blood stream of the rabbits injected at birth and during adult life with D.H.A. was examined (Table III). The elimination of H.A. was also studied in three rabbits (372A, B, C) which had been injected in the same manner as 146A, B, C with four doses of 10 mg. D.H.A. during the first 3 days of life, but which had not been injected with D.H.A. subsequently. In addition, the elimination of H.A. was measured in rabbits which had been injected with H.A. at birth.

Rabbits injected at birth with D.H.A. which had subsequently shown a more or less transient response to D.H.A. were found to eliminate H.A. more rapidly from their blood stream than other rabbits in the same group or than rabbits injected with H.A. at birth. The elimination of H.A. reflected the initial response of the rabbits. The three rabbits from litter 146 had shown a gradation of response to D.H.A.; 146A had responded with the highest titre and in every one of four courses of injections, 146B had formed antibody demonstrable by agglutination after the first and second course of injections and 146C only after the first course (Table II). In the subsequent studies of elimination circulating antigen was found in 146A on the 5th day after the last injection but not subsequently, in 146B on the 5th and 11th but not on the 18th day, and in 146C on the 5th, 11th and 18th day. Only in the serum of rabbit 146A was antibody detected (Table IV).

The half life of human albumin determined in 146C agreed with that determined in rabbits which had been injected with H.A. at birth. The other rabbits which had been injected with D.H.A. at birth eliminated H.A. at the same rate as rabbits injected with H.A. at birth, except 264D which had previously shown a relatively strong response to D.H.A. Elimination studies with H.A. have thus reflected the response to D.H.A. (Tables II and III); only a small proportion of the responding animals would have been recognised as such if the antibody response to H.A. (rather than elimination) had been determined. It remained questionable whether injection with H.A., not preceded by immunisation with

TABLE II.—*Antibody Response to Injections of D.H.A. of Rabbits Injected with D.H.A. at Birth and of Controls*

(*a*) Intraperitoneal injections immediately after birth.

| Litter | 102 | 146 | 263 | 264 | 372 |
|---|---|---|---|---|---|
| Individuals injected | E, F, G | A, B, C | C, E | C, D | A, B, C |
| Total quantity of D.H.A. (mg.) injected (at intervals of 24 hr.) | 1×5 | 4×10 | 20×4 | 20×1 | 4×10 |

(*b*) Dose injected into adult rabbits.

| Total mg. D.H.A. injected in each course (at intervals of 48 hr.) | 3×5 . 3×10 . 3×10 | 3×5 . 3×10 . 3×10 . 3×10 | 3×5 . 3×10 . 3×10 | 3×5 . 3×10 . 3×10 | 3×10‖ . 3×10 |
|---|---|---|---|---|---|

(*c*) Agglutination titre of tanned erythrocytes sensitised with D.H.A.

| Bleedings‡ : days after birth | | 77 | 97 | 124 | 77 | 97 | 128 | 305 | 77 | 97 | 138 | 77 | 97 | 128 | 186 | 205 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbits | A | * | * | * | **200** | **100** | **100** | **100** | § | § | § | § | § | § | <5 | <5 |
| | B | * | * | * | **100** | **50** | <5 | <5 | § | § | § | § | § | § | <5 | <5 |
| | C | * | * | * | **100** | <5 | <5 | <5 | <5 | <5 | <5 | **50?** | <5 | <5 | <5 | <5 |
| | D | § | § | § | § | § | § | § | § | § | § | <5 | **200** | 5 | 50,000 | 50,000 |
| | E | **1,000** | **10,000** | **20,000** | § | § | § | § | **10** | <5 | <5 | § | § | § | 5,000 | 10,000 |
| | F | **200** | **20,000** | **50,000** | † | † | † | 500 | — | — | — | § | § | § | **100?** | 5,000 |
| | G | **200** | **20,000** | **50,000** | 2,000 | 20,000 | 50,000 | 20,000 | § | § | § | § | § | § | § | § |
| | M | * | * | * | 2,000 | 50,000 | 100,000 | 50,000 | 500 | 20,000 | 50,000 | § | § | § | 20,000 | 50,000 |
| | 112 | 2,000 | 50,000 | 20,000 | | | | | | | | | | | | |
| | 114 | 2,000 | 20,000 | 20,000 | | | | | | | | | | | | |

* See Table V.
† Not injected.
‡ Bleedings : 11 days after the last injection of the first course and 5 days after the last injection of subsequent courses.
§ Not available.
‖ Injected with H.A. before injections with D.H.A. were given (see Table III).
Titres shown in heavy type have been measured in sera of rabbits injected at birth with D.H.A.
? Indicates the presence of a prezone.

TABLE III.—*The Elimination of H.A. from the Blood Stream of Rabbits Injected with H.A. at Birth, and from Rabbits Injected with D.H.A. at Birth*

Each adult rabbit received 3 i.v. injections with H.A. at intervals of 48 hr. and was bled 5 days, 11 days and sometimes 18 days after the last injection.

| Antigen injected immediately after birth | Litter | Individual | Day of last injection | Day of bleeding | H.A. in circulation $\mu$g./2 ml. | Half life of H.A. Individual values (measured between 5th and 11th day) | Half life of H.A. Mean | Half life of H.A. Standard error |
|---|---|---|---|---|---|---|---|---|
| None | | M | | 423 | — | | | |
| | | | | 429 | — | — | — | — |
| | | | | 436 | — | | | |
| | | A | | 423 | 13·25 | | | |
| | | | | 429 | — | — | — | — |
| | | | | 436 | — | | | |
| | 146 | | 418 | | | | | |
| | | B | | 423 | 5·0 | | | |
| | | | | 429 | 1·5 | 3·5 | — | — |
| | | | | 436 | — | | | |
| | | C | | 423 | 18·9 | | | |
| | | | | 429 | 8·0 | 4·8 | — | — |
| | | | | 436 | 3·25 | | | |
| | | D | | 201 | 23·25 | | | |
| | | | | 207 | — | — | — | — |
| | 264 | | 196 | | | | | |
| | | C | | 201 | 27·5, 30·0 | | | |
| D.H.A. | | | | 207 | 13·25, 15·0 | 5·7, 6·0 | | |
| | | C | 256 | 261 | 31·0 | | | |
| | | | | 267 | 14·75 | 5·6 | | |
| | 263 | | | 274 | 5·0 | | | |
| | | E | 207 | 212 | 24·25 | | | |
| | | | | 218 | 12·0 | 5·9 | 5·60 | 0·25 |
| | | A | | 104 | 17·5 | | | |
| | | | | 110 | 7·6 | 5·0 | | |
| | | | | 117 | 3·8 | | | |
| | | B | | 104 | 18·75 | | | |
| | 372 | | 99 | 110 | 8·5 | 5·3 | | |
| | | | | 117 | 4·25 | | | |
| | | C | | 104 | 15·25 | | | |
| | | | | 110 | 7·0 | 5·3 | | |
| | | | | 117 | 2·75 | | | |
| | | | 97 | 102 | 10·5 | | | |
| | | | | 108 | 3·75 | 4·0 | | |
| | 266 | A | 128 | 133 | 15·3 | | | |
| | | | | 139 | 7·4 | 5·7 | | |
| | | | 197 | 202 | 29·5, 30·0 | | | |
| H.A. | | | | 208 | 14·75, 13·75 | 6·0, 5·3 | 5·12 | 0·22 |
| | 88 | E | 208 | 213 | 9·9 | | | |
| | | | | 218 | 5·0 | 5·0 | | |
| | 693 | C* | 722 | 727 | 8·5 | | | |
| | | | | 733 | 4·0 | 5·5 | | |
| | 712 | C* | 657 | 662 | 8·5 | | | |
| | | | | 668 | 3·5 | 4·7 | | |

* For details of the injections given at birth see Cinader and Dubert (1955).

TABLE IV.—*The Response to H.A. of Animals Injected with D.H.A. at Birth and of Controls* (cf. Table III)

(*a*) Dose.

| | |
|---|---|
| Litter (individual rabbits) | 146 (**A, B, C,** F, G, M) |
| Injection with H.A., days after birth | 414 |
| | 416 |
| | 418 |
| Total mg. H.A. injected | 3 × 10 |

(*b*) Agglutination titre of tanned erythrocytes sensitised with H.A.

| Bleedings: days after birth | | 423 | 429 | 436 |
|---|---|---|---|---|
| Rabbits | M | 20,000 | 20,000 | 20,000 |
| | **A** | <5 | **100** | **50** |
| | **B** | <5 | <5 | <5 |
| | **C** | <5 | <5 | <5 |
| | F | 10,000 | 5,000 | 1,000 |
| | G | 5,000 | 2,000 | 1,000 |

Titres and letters in heavy type refer to rabbits injected with D.H.A. immediately after birth.

D.H.A., would have revealed a more rapid elimination of antigen. However, the rabbits (372 A, B, C) which had been injected with D.H.A. at birth but had not been subsequently injected with D.H.A., eliminated H.A. in a manner indistinguishable from that of animals injected after birth with an effective dose of H.A. Rabbits from litter 372 were subsequently injected with D.H.A. and did not form antibody to it (Table II). The experiment with litter 372 had thus failed to support but had not disproved the proposition that the difference in the response to H.A. and D.H.A. was due to the difference in the elimination of the two antigens from the circulation.

We have so far seen that the antibody response of rabbits injected with D.H.A. immediately after birth is completely or almost completely inhibited, that there is a strong variability in the extent of the feeble residual response within animals from different litters and that though the residual response declines during successive courses of immunisation it remains demonstrable by the rate of elimination of H.A. from the blood stream. It has been impossible to support the view that this difference between rabbits injected with H.A. and with D.H.A. at birth is due to the different rate of elimination of this antigen when administered during adult life. The rapid elimination of D.H.A. by a constitutive process may allow only a relatively small portion of this antigen to reach antibody forming cells.

It is likely that the different rate of elimination of H.A. and D.H.A. observed in adult normal rabbits would also be found in new-born animals, particularly since this difference is not due to an immune response (Cinader and Dubert, 1957). If this is so, a smaller percentage of the administered dose may be reaching potential antibody-forming cells when D.H.A. rather than H.A. is injected.

*Response during adult life to injections with D.H.A. of one rabbit injected with H.A. immediately after birth*

A small number of rabbits with acquired immunological tolerance to H.A. respond during adult life to injections with D.H.A. by the formation of an antibody (Cinader and Dubert, 1955 ; 1956). Another animal of this type was encountered in 266A (See Table V). The elimination of H.A. from the circulation of this rabbit

was similar to that of other rabbits which had acquired tolerance to H.A. (Table III). The rabbit did not respond demonstrably to the first but did respond to the second and third course of injections with D.H.A., the titre developed after the third course being considerably higher than after the second course (Table V). The agglutination by these sera of tanned cells sensitised with D.H.A. was readily inhibited by D.H.A. but was not inhibited by H.A. (Fig. 2). So far the antibody response to D.H.A. of rabbits injected at birth with D.H.A. and H.A. has been examined. The following experiments were designed to examine the antibody response to D.H.A. in rabbits injected at birth with other azo proteins.

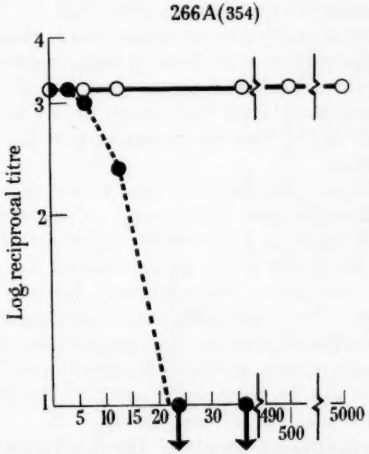



FIG. 2.—The inhibition by H.A. and D.H.A. of agglutination-erythrocytes sensitised with D.H.A. D.H.A. antiserum from a rabbit injected with H.A. at birth and later immunised with D.H.A. (Table IV).

Number in brackets gives age of the donor in days.

○——○ agglutination inhibited by H.A.

●- - -● agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates $\mu$g inhibiting Antigen/0·15 ml. Antiserum.

*Response during adult life to injections with D.H.A. of rabbits injected with D.R.S. or with D.Rn immediately after birth*

The introduction of azo groups into H.A. brought about a weight difference of only 3 per cent between native and chemically modified protein ; the principal portion of the H.A. and D.H.A. remained essentially similar. The percentage of dissimilar antigenic determinants becomes very much higher when two different modified proteins are compared ; in this instance, the difference is due to the protein moiety and hence in 97 per cent of the molecular weight of D.H.A.

The above considerations have no quantitative but only qualitative significance ; they neglect original similarities in amino acid sequences and changes in protein configuration which may be caused by the introduction of the diazo groups.

Coupling with diazonium salt may increase the similarity of two proteins in surface structure or spatial arrangement. Nevertheless the consideration in terms of weight may give some indication of the considerable difference between the system investigated at present and that described in earlier publications.

The injections of D.Rn and D.R.S. given to new-born rabbits within the first days after birth are shown in Table V. The quantity of D.R.S. which was injected into new-born rabbits exceeded the quantity of purified proteins injected into other rabbits so that the amount of azo rabbit albumin contained in the mixture of rabbit proteins should be similar to the weight of the purified proteins.

The rabbits injected immediately after birth and their litter mates received during adult life three courses of injections with D.H.A. The response to D.H.A. of animals injected at birth with azo proteins other than D.H.A. did not differ from that of the controls (Table V). Thus a very marked difference was found between the results obtained here and those observed in the response to D.H.A. of rabbits injected immediately after birth with H.A., when the majority of the rabbits had been found to be equally unresponsive to the native and to the chemically modified protein.

It remained to examine qualitatively the response in the rabbits injected with D.Rn or D.R.S. at birth and in controls. It seemed possible that animals injected with D.Rn or D.R.S. at birth might have formed an antibody entirely adapted to the protein moiety of H.A., while animals not injected at birth would have formed antibody adapted to the azo group as well as antibody adapted to the protein determinants. Two sera differing in this way could be distinguished by inhibition with H.A. Were the antibodies exclusively adapted to H.A., inhibition with H.A. would completely abolish the reaction of tanned cells sensitised with D.H.A. This would not be the case if some of the antibody molecules were primarily adapted to the sulphanilic acid group.

A number of control animals received three courses of immunisation with D.H.A. The agglutination by the resulting D.H.A. antisera of erythrocytes sensitised with D.H.A. was completely inhibited by the prior addition of D.H.A. to the antisera but only partially inhibited by the addition of H.A. A plot of reciprocal agglutination titre as a function of the quantity of H.A. added to a D.H.A. antiserum descends steeply and finally reaches a more or less constant level (Fig. 3). For D.H.A. antisera from animals not injected at birth, the initial portion of steep descent did not reach titres of less than 1/10 (Fig. 3). An exception to this was found in the antibody from a rabbit which had been immunised with H.A. before being immunised with D.H.A. The D.H.A. antisera considered in the following comparison were obtained from rabbits which had not been injected with H.A. before being immunised with D.H.A. Some D.H.A. antisera from controls were completely inhibited by H.A. in extreme antigen excess* (2000–3000 $\mu$g./0·15 ml.) While this is encountered quite frequently after the first course of immunisation, sera obtained from controls after two to three courses of immunisation gave a residual titre even in the presence of a vast excess of antigen.

The difference between litters was tested in a one-way classification analysis of variance, comparing the variation between litters and within litters. The difference between litters was found to be more marked at higher than at lower levels of inhibiting H.A. For this reason the subsequent analysis was carried out on litter means. Differences between groups were only accepted as significant if significant differences could be demonstrated within a group of animals from

TABLE V.—*Antibody Response to Injections with D.H.A. of Controls and of Rabbits Injected at Birth with D.Rn* (7), *D.Rn* (18) *and D.R.S. and of one Rabbit* (266A) *Injected at Birth with H.A.*

| | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Litter | | | | | 102 | | 194 | | | | | | 266* | | | | 262 | | | 373 | | | | | |
| Individuals injected | | | | | A, B | | B, C, D, E | | | | | | C, E | | | | D | | | A, B, C, D | | | | | |
| Antigen injected | | | | | D.Rn (7) | | D.Rn (18) | | | | | | D.Rn (18) | | | | D.R.S. | | | D.R.S. | | | | | |
| Total quantity (in mg.) injected (at intervals of 24 hr.) | | | | | 1×5 | | 4×10, then 15×1 | | | | | | 20×1 | | | | 20×4 | | | 20×20 | | | | | |
| | | | | | | | (*b*) Dose injected into adult animals. | | | | | | | | | | | | | | | | | | |
| Total wt. of D.H.A. injected (at intervals of 48 hr.) | | | | | 3×10 | 3×10 | 3×5 | 1×10 | 3×10 | | 1×10 | | 3×5 | 3×10 | | 3×10 | 3×5 | 3×10 | 3×10 | 2×5 | 3×10 | 3×10 | | | |
| | | | | | | | (*c*) Agglutination titre of tanned erythrocytes sensitised with D.H.A. | | | | | | | | | | | | | | | | | | |
| Bleedings‡ : days after birth | | | | | 296 | 316 | 220 | 237 | 407 | 417 | 430 | | 303 | 323 | 329 | 354 | 79 | 97 | 138 | 81 | 102 | 133 | | | |
| Rabbits | A | | | | **200** | **20,000** | § | § | § | § | § | | <5* | 200* | 100* | 2,000* | § | § | § | **500** | **10,000** | **10,000** | | | |
| | B | | | | **2,000** | **20,000** | **1,000** | **10,000** | **20,000** | § | † | | § | § | § | § | § | § | § | **500** | **20,000** | **5,000** | | | |
| | C | | | | § | § | **500** | **5,000** | **10,000** | § | † | | **1,000** | **10,000** | **5,000** | **10,000** | § | § | § | **200** | **20,000** | **20,000** | | | |
| | D | | | | § | § | **5,000** | **10,000** | **5,000** | § | † | | § | § | § | § | **10,000** | **50,000** | **50,000** | 50 | 20,000 | 10,000 | | | |
| | E | | | | § | § | **2,000** | **10,000** | **20,000** | § | † | | **1,000** | **10,000** | **5,000** | **20,000** | § | § | § | 500 | 20,000 | 20,000 | | | |
| | F | | | | § | § | † | † | <5 | 100 | 10,000 | | § | § | § | § | § | § | § | § | § | § | | | |
| | M | | | | 200 | 50,000 | 5,000 | 10,000 | 10,000 | § | † | | 2,000 | 20,000 | 20,000 | 20,000 | 10,000 | 10,000 | 200,000 | 500 | 50,000 | 20,000 | | | |
| | 110 | | | | 1,000 | 20,000 | | | | | | | | | | | | | | | | | | | |
| | 111 | | | | 1,000 | 20,000 | | | | | | | | | | | | | | | | | | | |

* 266A injected with H.A. at birth (4×10 mg. at intervals of 24 hr.).
† Not injected.
‡ Bleedings : 11 days after the last injection of the first course and 5 days after the last injection of each subsequent course.
§ Not available.
‖ Injected with ribonuclease at birth.
Titres shown in heavy type have been measured in sera from rabbits injected at birth with D.Rn or D.R.S.

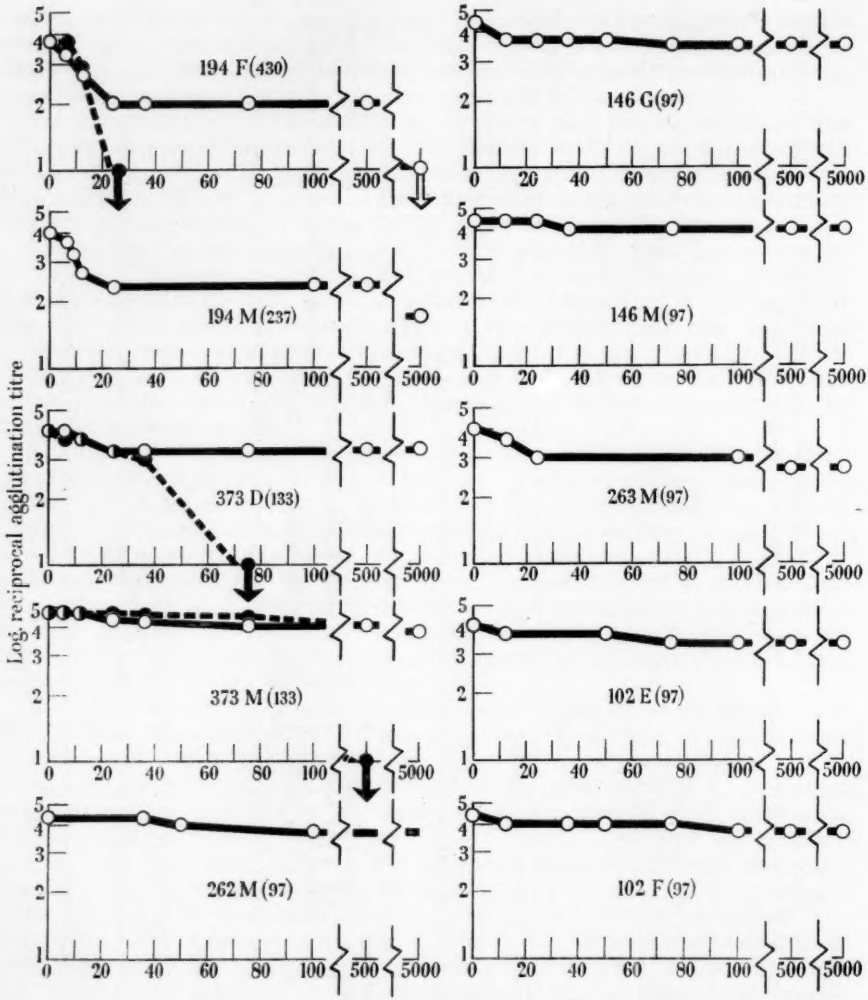



FIG. 3.—Controls.

The inhibition by H.A. (and D.H.A.) of agglutination-tanned erythrocytes sensitised with with D.H.A.

D.H.A. antisera from adult normal animals not injected with D.H.A. at birth and from rabbits 102E and 102F which received a single injection of 5 mg. D.H.A. immediately after birth, a dose which does not affect their subsequent response (see Table II). Values in brackets give the age of the donor animal, except in the case of the mothers (M) where the number in brackets refers to the days after the birth of their litter.

○——○ agglutination inhibited by H.A.

●- - -● agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates $\mu$g inhibiting Antigen/0·15 ml. Antiserum.

the same litter as well as between groups of animals derived from different litters. The results of inhibition-agglutination with sera from the control group were compared with those with the D.H.A.-antisera from animals injected with

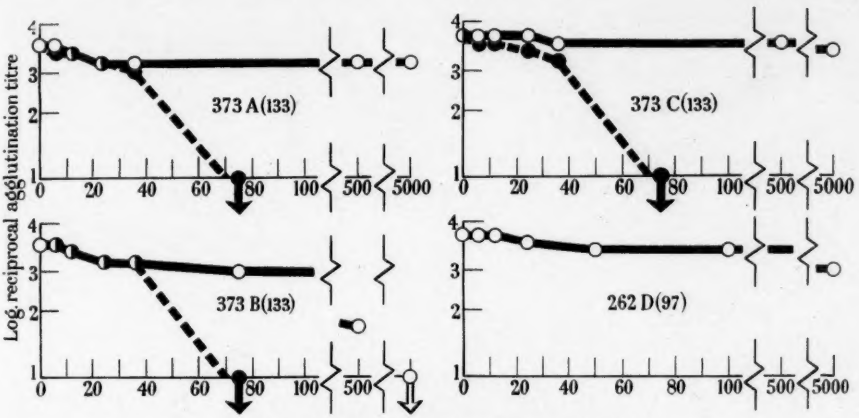



FIG. 4.—Rabbits injected with D.R.S. at birth.

The inhibition by H.A. (and D.H.A.) of agglutination-tanned erythrocytes sensitised with D.H.A.

D.H.A. antisera from adult animals injected with D.R.S. at birth (see Table V). Values in brackets give the age of the donor animal.

○——○ agglutination inhibited by H.A.

●- - -● agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates $\mu$g inhibiting Antigen/0·15 ml. Antiserum.

D.R.S. at birth (Fig. 4) and with those from animals injected with D.Rn (18) at birth (Fig. 5). The variable considered was :

$$x_n = \log (t_n/t_o) \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where $t_n$ = titre with $nx$ $\mu$g. of inhibiting H.A. and $t_o$ = titre without inhibiting H.A.; $x = \frac{t_o}{10{,}000}$, $n$ = $\mu$g. protein/0·15ml. serum.

It can be seen from Table VI that the antibody from rabbits injected immediately after birth with D.R.S. did not differ from that of controls. The D.H.A. antibody from rabbits injected at birth with D.Rn (18) appeared to be more effectively inhibited by H.A. than that of controls (Fig 5). This became significant in slight antigen excess, corresponding to fairly low concentrations of inhibiting H.A. (Table VIa). The significance of this difference was also tested within a group of rabbits from the same litter (194) by computing the difference between the $x_n$ (1) of controls and of animals injected with D.Rn (18) at birth. The regression of this difference on the logarithm of the inhibiting dose was significant on the 5 per cent level (Table VIb). If the regression of $\Delta x_n$ (D.Rn-control) was

determined against 1, 2, 3, 4, 5 (representing the different values of $nx$), it was found to be significant at the 1 per cent level ($F = 50{\cdot}83$ on 1, 3 degrees of freedom). On the other hand, a similar comparison between rabbits injected at birth with D.R.S. and controls from litter 372 and 262 showed the regression to be not significant (Table VIb). The comparison within litters and between litter means

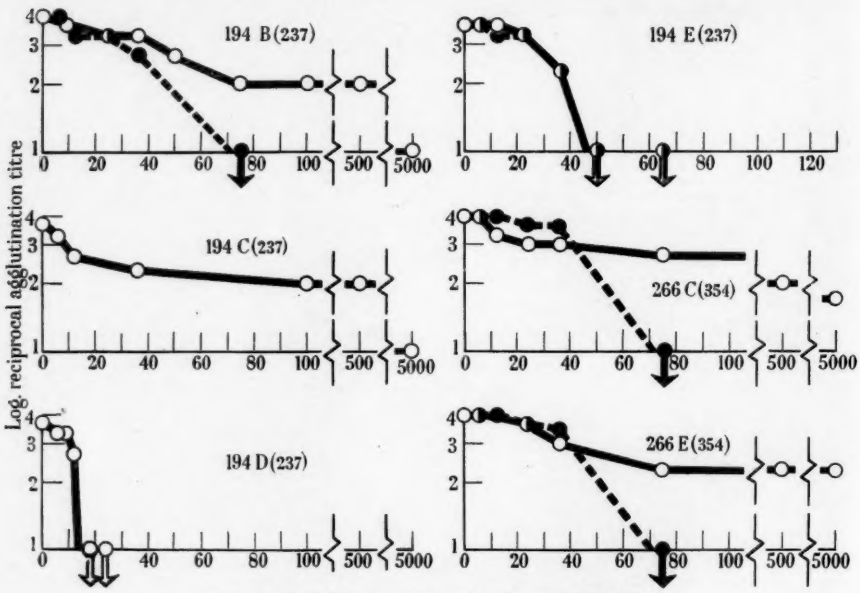



FIG. 5.—Rabbits injected with D.Rn (18) at birth.

The inhibition by H.A. (and D.H.A.) of agglutination-tanned erythrocytes sensitised with D.H.A.

D.H.A. antisera from adult animals injected with D.Rn (18) at birth (see Table V). Values in brackets give the age of the donor animal.

agglutination inhibited by H.A.

agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates $\mu$g inhibiting Antigen/0·15 ml. Antiserum.

showed that D.H.A. antibody from rabbits injected immediately after birth with D.R.S. did not differ from antibody of the controls and that D.H.A. antibody from rabbits injected immediately after birth with D.Rn (18) differed from antibody of controls.

A difference between the antibodies of controls and of rabbits injected at birth could not be demonstrated by inhibition of agglutination with D.Rn or D.R.S. Inhibition of agglutination was never greater than one dilution step and was therefore within the limits of error of the method.

TABLE VI.—*The Inhibition of D.H.A. Antisera from Rabbits Injected Immediately after Birth with D.R.S., D.Rn* (18) *and from Controls (Test of Significance)*

The variation of $x_n$* with inhibiting dose

(*a*) Comparison of litter means.

| Inhibiting dose $n$ ($\mu$g. H.A./0·15 ml.) | | 10 | 20 | 40 | 80 | 500 |
|---|---|---|---|---|---|---|
| $x_n$ | Control | 0·304 | 0·584 | 0·758 | 0·850 | 0·913 |
| | D.R.S. at birth | 0·172 | 0·500 | 0·590 | 0·633 | 0·967 |
| | D.Rn (18) at birth | 0·271 | 0·541 | 1·277 | 1·889 | 2·312 |
| Comparison of litter means | Controls and rabbits injected with D.R.S. at birth: $t>$ | 0·924 | 0·311 | 0·490 | 0·608 | 0·137 |
| | Controls and rabbits injected with D.R.S. at birth: $>P>$ | 0·4, 0·3 | 0·8, 0·7 | 0·7, 0·6 | 0·6, 0·5 | 0·9, 0·8 |
| | Controls and rabbits injected with D.Rn (18) at birth: $t>$ | 0·231 | 0·158 | 1·511 | 2·914 | 3·528 |
| | Controls and rabbits injected with D.Rn (18) at birth: $>P>$ | 0·9, 0·8 | 0·9, 0·8 | 0·2, 0·1 | 0·05, 0·02 | 0·01, 0·001 |

(*b*) Comparison within litters.

| Inhibiting dose $n$ ($\mu$g. H.A./0·15 ml.) | | 10 | 20 | 40 | 80 | 500 |
|---|---|---|---|---|---|---|
| $x_n$ | Controls (from litters 373, 262) | 0·180 | 0·375 | 0·585 | 0·650 | 0·687 |
| | D.R.S. (from litters 373, 262) | 0·172 | 0·500 | 0·590 | 0·633 | 0·967 |
| $x_n$ (D.R.S.) $- x_n$ (controls) as a function of log $n$ | $\Delta x_n$ | −0·008 | 0·125 | 0·005 | −0·017 | 0·279 |
| | regression of $\Delta x_n$ on log $n$ | *Not significant* ($F = 2·86$; 1, 3 degrees of freedom) | | | | |
| $x_n$ | Controls (from litter 194) | 0·640 | 1·250 | 1·600 | 1·700 | 1·700 |
| | D.Rn (18) (from litter 194) | 0·252 | 0·523 | 1·510 | 2·277 | 2·775 |
| $x_n$ (D.Rn (18)) $- x_n$ (control) as a function of log $n$ | $\Delta x_n$ | −0·388 | −0·682 | −0·090 | 0·577 | 1·075 |
| | regression of $\Delta x_n$ on log $n$ | *Significant* at the 5 per cent level ($F = 19·05$; 1, 3 degrees of freedom) | | | | |

*$x_n = \log (t_n/t_o)$, where $t_n$ = titre in the presence of $nx$ $\mu$g. of H.A., $n = \mu$g. of inhibiting H.A./0·15 ml. of serum, $x = t_o/10·000$, $t_o$ = titre in absence of inhibitor.

*The response to D.Rn* (18) *of animals injected immediately after birth with D.H.A. and of controls*

In the preceding experiments rabbits had been injected with D.R.S. and D.Rn (18) immediately after birth, and their subsequent response to D.H.A. had been found to differ quantitatively very little from that of controls. We had no direct information on the effect of the injection on the response of the adult animal to the antigen D.R.S. and D.Rn respectively. Thus a piece of evidence important for the evaluation of results was missing. Since information existed on the adult response to D.H.A. of rabbits injected with D.H.A. at birth, the injection of these rabbits with another protein coupled with *p*-diazo-benzene-sulphonic acid was of particular interest.

Ribonuclease which, administered intravenously, had been a fairly good antigen in wild rabbits was a very bad antigen in white Himalayans. It was therefore not surprising that repeated courses of injections with D.Rn (18) given to normal white Himalayans failed to produce an antibody response in six out of seven normal animals.

In spite of these poor prospects, animals from two litters of white Himalayans, some of which had been injected with D.H.A. at birth and whose response to D.H.A. is recorded in Table II, were given courses of injections with D.Rn (18). Most of them formed an antibody which agglutinated tanned cells sensitised with D.Rn (7) (Table VII). After the fourth course of injections with D.Rn (18) had been given to the rabbits of litter 146, two controls and one of the animals injected at birth with D.H.A. had failed to form an antibody against D.Rn. It was thought possible that this relatively high degree of responsiveness was connected with the previous immunisation with D.H.A. This was supported by the unresponsiveness of rabbit 146F, the only rabbit of litter 146 not previously immunised with D.H.A., but contradicted by the unresponsiveness of the control 146G and of 146A which had given, though injected at birth with D.H.A., the highest and most persistent response to D.H.A. when compared with his similarly injected litter mates 146B and C.

TABLE VII.—*The Response to D.Rn* (18) *of Some Rabbits Injected at Birth with D.H.A. and Controls*

All the animals received several courses of injection with D.H.A. during their adult life and before they were injected with D.Rn.

(*a*) Intraperitoneal injections immediately after birth.

| Litter | 146 | | | | | | | | | | | 263 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Details of injection | (See Table II) | | | | | | | | | | | | |
| (*b*) Dose injected into adult rabbits. | | | | | | | | | | | | | |
| Total mg. D.Rn (18) injected (at intervals of 48 hr.) | 3×5 | 3×4·5 | | 3×5 | | 1×8 | | 3×10 D.H.A. | 3×5 | 2×10 | | 3×5 | 3×10 |

(*c*) Agglutination titre of tanned erythrocytes sensitised with D.Rn (7).

| Bleedings†: days after birth | | 205 | 225 | 262 | 284 | 286* | 289 | 295 | 305 | 374 | 491 | 330 | 366 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbits | A | <5 | <5 | <5 | <5 | <5 | <5 | <5 | <5 | 200 | 200 | § | § |
| | B | <5 | 200 | 2000 | 5000 | <5 | 200 | 50 | 20 | 1000 | 5000 | § | § |
| | C | <5 | <5 | <5 | <5 | <5 | 200 | 500 | <5 | 200 | 500 | 50 | 500 |
| | E | § | § | § | § | § | § | § | § | § | § | <5 | 20 |
| | F | <5 | <5 | <5 | <5 | <5 | <5 | <5 | <5 | <5 | <5 | § | § |
| | G | <5 | <5 | <5 | <5 | <5 | <5 | <5 | <5 | 50 | 50 | § | § |
| | M | <5 | 200 | 500 | <5 | <5 | <5 | 200 | 10 | 1000 | 1000 | <5 | <5 |

* Injection : 284 days after birth.

† Bleedings : 11 days after the last injection of the first course and 5 days after the last injection of each subsequent course.

§ Not available.

Titres in heavy type have been determined on bleedings from rabbits injected with D.H.A. immediately after birth.

A course of injection with D.H.A. was, therefore, given to all the rabbits of litter 146 and the following observations were made :

1. The injection with D.H.A. did not demonstrably affect the titre to D.Rn.

2. A subsequent course of injections with D.Rn produced a response in 146F which was so transient and so weak as to be negligible.

3. 146G and 146A showed a response to D.Rn after a subsequent course of injections with D.Rn.

The last of these observations could not be linked with the preceding injections with D.H.A. since 146A and 146G had received and responded to numerous injections with D.H.A. before the injections with D.Rn (18) had been started. Furthermore the antibody to D.H.A. formed by 146G before the first courses with D.Rn had been given was analysed by agglutination-inhibition and had been demonstrated to contain a high percentage of antibody to D.H.A. which could not be inhibited by H.A. but only by D.H.A.

The first and the second observations, though not excluding the view that the response to D.Rn might be in some way mediated by a preceding response to D.H.A., did not support it. It was finally abandoned when it was found impossible to elicit a response to D.Rn (18) in rabbits from litter 194 when this antigen was injected after a preceding course of injections with D.H.A. Moreover it was found in litter 263 that rabbit 263C, which had been shown to have acquired complete immunological tolerance to D.H.A., responded nevertheless to D.Rn (18).

On balance these experiments can thus be taken to support the previous conclusion that the injection immediately after birth of an azo protein containing 3 per cent azo groups does not inhibit antibody response to another azo antigen which is injected during adult life.

It is difficult to arrive at a reliable qualitative estimate of the antibody specificity of these antisera to D.Rn (18). Agglutination-inhibition has to be carried out with tanned cells sensitised with D.Rn (7). Thus only a fraction of the total antibody can be considered. Inhibition of this agglutination by Rn and D.Rn (18) respectively did not show a difference between the control and the animals injected with D.H.A. immediately after birth (Fig. 6). There was therefore no evidence that injection of D.H.A. during the first days of life had any effect on the response to D.Rn (18) of the adult rabbit.

## DISCUSSION

Acquired immunological tolerance has been defined by Billingham, Brent and Medawar (1953, 1955) as a primary failure of the machinery of immunological response. It can be induced by the maintenance of a critical level of foreign proteins during the first days after birth (Hanan and Oyama, 1954 ; Dixon and Maurer, 1955 ; Cinader and Dubert, 1955).

A number of recent publications have reported unsuccessful attempts to induce acquired tolerance to foreign antigens. To evaluate such findings the following factors may have to be considered : (1) The quantity of single antigens injected. (2) The number, route and time of injections. (3) The method of assay of the antibody, and the ability of the assay to distinguish between different antigens that may be injected in a mixture and of which some but not all may have induced tolerance, and possibly (4) the structural similarity of the foreign antigens to homologous macromolecules of the injected animal (cf. Billingham and Brent, 1956).

Our finding that a single injection of 5 mg. of H.A. within the first twelve hours after birth did not lead to the acquisition of immunological tolerance may appear to conflict with the report of Dubert and Paraf (1957) who observed that immunological tolerance was acquired when a much smaller total quantity of H.A. was injected in daily doses during the first eight days of life. Though genetic differences between strains of rabbits might account for these discrepancies,

it is equally probable that to establish acquired immunological tolerance a fairly constant level of antigen has to be maintained within the body fluids during the first week after birth so that repeated injections of antigen might be much more effective than a single injection of the same quantity.

Most of the reported failures to induce tolerance were met with complex mixtures of antigens, foreign erythrocytes or bacteria. The heterogeneity of the antigen may contribute to negative results. In cellular material the usual assay of antibody depends on a small number of the many antigens contained

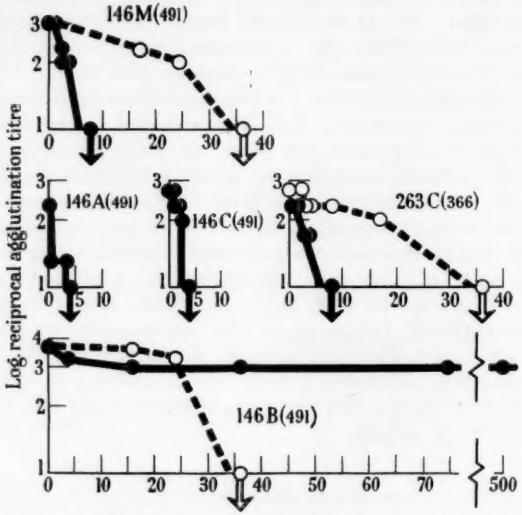



FIG. 6.—The inhibition by Rn and D.Rn (18) of agglutination-erythrocytes sensitised with D.Rn (7).

D.Rn (18) antisera from a normal rabbit (146M) and from rabbits injected at birth with D.H.A. (see Tables II and VII). Values in brackets give the age of the donor in days except for the mother (M) where the number in brackets gives the days after birth of her litter.

●---● agglutination inhibited by Rn.

○—○ agglutination inhibited by D.Rn (18).

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates $\mu$g inhibiting Antigen/0·15 ml. Antiserum.

in the cell. The only antigens reactive in an agglutination test, for instance, are those present on the surface of the cells and representing a minute fraction of the total weight injected. Moreover the weight of cellular material injected into the new-born animals was frequently chosen with reference to the immunising dose of the antigen. The immunising dose may, however, not bear any relation to the dose conferring acquired tolerance. It appears unlikely to do so, if it is considered that whilst immunisation can be effective if only a small number of antibody-forming cells encounter the antigen, acquired tolerance is, for the same reason, best demonstrated if the vast majority of the antibody-forming cells of a new-born animal encounter the antigen. In spite of these reservations, it is

conceivable that the similarity of a foreign protein to the proteins of the recipient affects the ability of the foreign protein to cause acquired immunological tolerance. It is, therefore, of some interest that an azo protein (D.H.A.) may give rise to acquired tolerance.

The acquired immunological tolerance to D.H.A. was not always complete; the most distinctive feature of the feeble residual response was its transient nature as judged by agglutination tests. Repeated injections into normal animals led to a gradual increase of antibody response, and after several courses of injections to a stabilisation or relative decline of the response. In the partially tolerant animal the peak response usually followed the first course of injections, subsequent courses eliciting less and less agglutinating antibody. Though this observation may seem reminiscent of immune paralysis it would be hazardous to identify it with this phenomenon since elimination studies with H.A. have shown the persistence of a weak immunological response. Thus a change in properties of antibodies rather than inhibition of antibody formation may have occurred during prolonged immunisation.

The small difference in responsiveness to H.A. of animals injected with H.A. at birth and to D.H.A. of animals injected with D.H.A. at birth may be attributable to the removal of D.H.A. by some constitutive process and by the relatively small dose of D.H.A. which reaches the cells mediating the acquisition of tolerance. Should this be the explanation, we would expect that the difference would be apparent and not real and that the injection of small doses of H.A. would produce an effect similar to that described for D.H.A. This feeble response to H.A. could only be demonstrated by elimination studies and not by titration of antibody. The prolonged persistence of H.A. in the circulation would mask the type of response to D.H.A. detected by agglutination tests in the serum of adult animals which had been injected with D.H.A. immediately after birth.

We have so far discussed the response to D.H.A. of adult animals which had been injected immediately after birth with D.H.A. It remains to consider the effect on the later response to D.H.A. of the injection into new-born animals of antigens sharing some chemical determinant with D.H.A. Three different responses were encountered by comparison with D.H.A. antibody from normal controls:

1. In rabbits injected with D.R.S. at birth neither a decrease in the titre nor a change in the specificity of D.H.A. antibody could be demonstrated.

2. In rabbits injected with D.Rn (18) at birth there was no decrease in the titre of D.H.A. antibody, there was, however, a slight but significant effect on specificity (Figs. 3 and 5).

3. In rabbits injected with H.A. at birth the antibody to D.H.A. differed in titre and specificity; the antibody was mainly adapted to the modified part of the protein (Fig. 2).

The difference between the effect of D.Rn (18) and D.R.S. could be due either to the protein moiety or to the chemical modification.

The similarity between H.A. and D.H.A., the two antigens differing by only 3 per cent of their weight, accounts for the profound effect of the injections of H.A. at birth on the response to D.H.A. of the adult animal.

It may be assumed that

(*a*) Immunological tolerance is acquired to homologous proteins during the normal development of an animal.

(*b*) This naturally-acquired tolerance influences the adult response to heterologous antigens to the extent to which the foreign antigen shares antigenic determinants with any homologous protein, thus limiting the number of effective determinants of most heterologous antigens.

Natural proteins have several determinants, if only a few determinants were shared between homologous and heterologous protein this would be reflected by the specificity of the antibody, if many or most groups were shared the foreign protein would be a " poor " antigen.

The elimination of H.A. from the blood stream of rabbits was determined between the 5th and 11th day after the last of three injections. Rabbits which had acquired immunological tolerance to H.A. or D.H.A. eliminated H.A. with a half life* of $5{\cdot}12 \pm 0{\cdot}22$ and $5{\cdot}6 \pm 0{\cdot}25$ days. The values determined by immunochemical methods are in good agreement with values ($5{\cdot}40 \pm 0{\cdot}25$) obtained in tolerant animals in which the elimination of $I^{131}$-H.A. had been followed by measuring radioactivity and is considerably higher than the value of $3{\cdot}95 \pm 0{\cdot}22$ days found in normal animals (Cinader and Dubert, 1956). The half life of human albumin (prepared by fractionation with ether) in normal rabbits is confirmed independently by a recent measurement ($4{\cdot}1 \pm 0{\cdot}4$ ; Weigle, 1957 ; HA prepared by fractionation with alcohol).

The difference between the half life of H.A. in normal and in tolerant rabbits may indicate the possible existence of a specific process of elimination which acts before the final rapid elimination of antigen by antibody begins. This process may be abolished by injection of the antigen immediately after birth. Half lives of foreign proteins may have to be determined in tolerant animals if they are to indicate the rate of elimination in the absence of immune processes and if they are to be comparable with half lives of homologous proteins. These conclusions must remain tentative until a satisfactory explanation has been found for variations in half lives determined in different laboratories in similar proteins prepared by different methods (solvent and salt-fractionation).

## SUMMARY

A single injection of 5 mg. of human albumin or of 5 mg. of azo human albumin given to rabbits within 12 hr. after birth did not induce acquired immunological tolerance. Acquired immunological tolerance to D.H.A. was induced by four daily injections of 10 mg. D.H.A. or by 20 daily injections of 1 mg. D.H.A. starting within 12 hr. after birth. The elimination of H.A. from the blood stream of animals tolerant to H.A. and D.H.A. was measured by precipitin tests and was found to be $5{\cdot}1 \pm 0{\cdot}2$ and $5{\cdot}6 \pm 0{\cdot}25$ days. The mean of the half life of H.A. measured by immunological methods and by radioactive methods in animals with acquired tolerance to H.A. and D.H.A. was $5{\cdot}35 \pm 0{\cdot}14$, a value considerably higher than that observed in normal animals. A very feeble antibody response to injections with D.H.A. during adult life was observed in some rabbits which had acquired immunological tolerance to D.H.A. This response as measured by agglutination decreased and usually disappeared during subsequent courses of immunisation but could still be demonstrated by the increased rate of elimination of H.A. from the blood stream. An animal tolerant to H.A. was found to respond to D.H.A.

* The half life of rabbit albumin (prepared by fractionation with ethanol) in rabbits has been measured as $5{\cdot}7 \pm 0{\cdot}3$ by Dixon, Maurer and Deichmiller (1953).

by the formation of antibody primarily adapted to the modified determinants. This brings the total of such responders (Cinader and Dubert, 1956) to three out of eight animals. Rabbits injected at birth with D.R.S. or D.Rn responded during adult life to D.H.A. with an antibody titre similar to that of animals not injected at birth.

Rabbits injected with D.Rn at birth produced antibodies to D.H.A. which were slightly less well adapted (significant at the 5 per cent level) to the modified determinants of the antigen than antibodies produced by the controls.

Thanks are due to Dr. R. C. Campbell (Statistics Group, Agricultural Research Council) for statistical advice and computations ; to Dr. J. M. Dubert for drawing our attention to the existence of D.H.A. antisera inhibited by H.A. in extreme antigen excess ; to the Armour Laboratories, Chicago, Illinois, for a gift of crystallised ribonuclease and to Dr. M. E. Mackay, Lister Institute, London, for preparations of human albumin. Sulphanilic acid, $S^{35}$, was supplied by the Radiochemical Centre, Amersham ; we are indebted to them for the analysis of this material.

## REFERENCES

Billingham, R. E. and Brent, L.—(1956) *Proc. roy. Soc.* B., **146**, 78.
*Iidem* and Medawar, P. B.—(1953) *Nature*, **172**, 603.—(1955) *Ann. N.Y. Acad. Sci.*, **59**, 409.
Borduas, A. G. and Brabar, P.—(1953) *Ann. Inst. Pasteur*, **84**, 903.
Boyden, S. V.—(1951) *J. exp. Med.*, **93**, 107.
Cinader, B.—(1957*a*) *Annu. Rev. Microbiol.*, **11**, 371.—(1957*b*) *Brit. J. exp. Path.*, **38**, 362.
*Idem* and Dubert, J. M.—(1955) *Brit. J. exp. Path.*, **36**, 515.—(1956) *Proc. roy. Soc.* B., **145**, 109.
*Idem* and Pearce, J. H.—(1956) *Brit. J. exp. Path.*, **37**, 541.
*Idem*, Rondle, C. and Pearce, J. H.—(1955) ' 3rd Int. Congr. Biochem.', 14–43.
Danysz, J.—(1902) *Ann. Inst. Pasteur*, **16**, 33.
Dixon, F. J., Maurer, P. H.—(1955) *J. exp. Med.*, **101**, 245.
*Iidem* and Deichmiller, M. P.—(1953) *Proc. Soc. exp. Biol., N.Y.*, **83**, 287.
Dubert, J. M. and Paraf, A.—(1957) *C.R. Acad. Sci.*, **244**, 686.
Hanan, R. Q. and Oyama, J.—(1954) *J. Immunol.*, **73**, 49.
Medawar, P. B.—(1956) *Proc. roy. Soc.* B., **146**, 1.
Weigle, W. O.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **94**, 306.

# THE SELECTIVE CUTANEOUS RESPONSE TO AUTOLOGOUS AND NON-AUTOLOGOUS PLASMA IN DOGS: OBSERVATIONS ON THE EFFECTS OF INBREEDING AND OF IMMATURITY

J. Q. BLISS*, P. B. STEWART† AND J. L. FULLER

*From the Department of Physiology, McGill University, Montreal, and the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

Received for publication October 3, 1957

THE skin of mongrel dogs responds selectively to autologous and homologous non-autologous plasma on first exposure. Intradermal injection of another dog's plasma causes a marked local increase in capillary permeability with distinct wheal formation, while a similar injection of the animal's own plasma has no such effect. This phenomenon was observed by Freeman and Schecter (1942) and by Bliss and Stewart (1957); the latter workers described it in detail and provided evidence that the increase in permeability was due at least in part to a release of endogenous histamine.

This peculiar property of mongrel dogs' plasma has several interesting implications. It shows that the generally accepted concept that homologous plasma is as innocuous to an animal as its own plasma is not always valid. Experiments with homologous plasma, particularly in dogs, must therefore be interpreted with some caution. Furthermore, the phenomenon requires that an inherent "marker" of individuality must be present in mongrel dogs' plasma, a feature analogous in some respects to the individual specificity encountered in tissue homografting and recently (MacKay, Larkin and Burnet, 1957) in the sera of a small number of patients with certain chronic diseases.

Bliss and Stewart (1957) postulated that the phenomenon might be attributed to gross ontogenetic disparities among mongrel dogs, with the protein of non-autologous plasma differing sufficiently from that of autologous plasma to act as a foreign protein. On this basis, its effect on dog skin might be analogous to the capillary-damaging effect of heterologous plasma described by Lake, Simmonds and Steinbeck (1953), or even to the histamine-releasing action of egg white described by Schachter and Talesnik (1952) in the skin of the non-sensitized cat.

It was decided, therefore, to examine the reactions of partially inbred dogs of standard pure breeds to plasma from their close relatives. If the non-autologous plasma effect were due essentially to ontogenetic differences among mongrel dogs, it should be greatly reduced or absent among closely related animals. In addition, this type of experiment should provide a finer assessment of the degree of specificity exhibited by dog plasma.

During these experiments, it was noted that plasma from a puppy produced no response in an adult recipient. Since this was the first instance we had encountered among dogs in which non-autologous plasma failed to produce a response, the activity of plasma from puppies was investigated in some detail.

* Medical Research Fellow, National Research Council, Canada.
† Medical Research Associate, National Research Council, Canada.

## METHODS

*Inbred dogs*

Animals from the dog colony of the Roscoe B. Jackson Memorial Laboratory were studied. The majority of experiments were conducted on the pure-bred lines of Basenjis and Springer Spaniels whose pedigrees are shown in Fig. 1. These lines have been inbred for several generations, though not long enough to produce the degree of homozygosity found in strains of inbred mice and rats. All animals tested were adults. None of these animals had previously been exposed to one another's plasma, serum or tissue. All of them, however, at 8 and 10

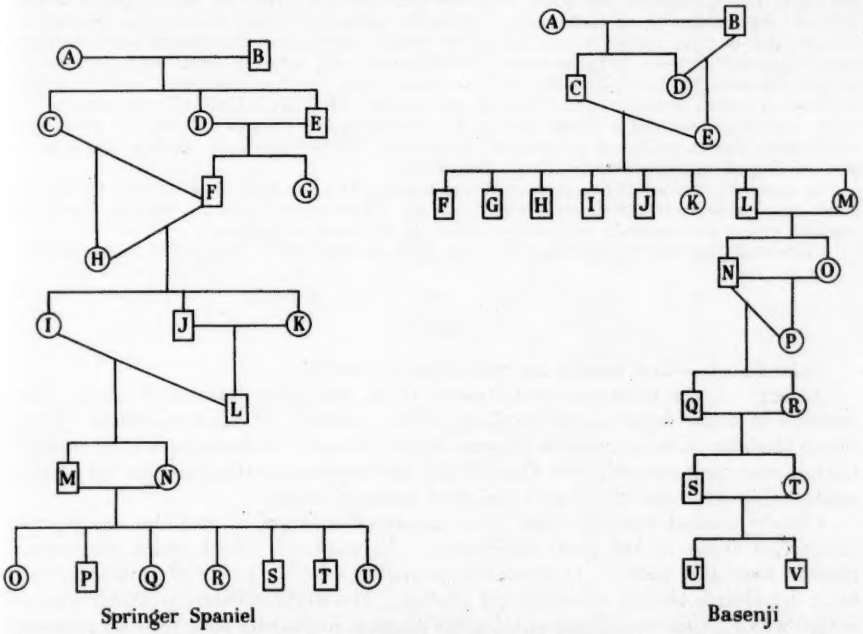



FIG. 1.—Pedigrees of the Basenji and Springer Spaniel inbred lines. Basenjis A and B were pure-bred, and partially related. Springer Spaniels A and B were pure-bred siblings. Note that neither pedigree has any outcross. Horizontal connections represent full-sib matings; diagonal connections represent back-crosses to parent or to parent's sibling.

weeks of age, had received anti-distemper serum prepared and pooled from the dogs of a commercial producer of veterinary products and anti-hepatitis vaccine prepared from dog liver from the same source.

Five ml. blood samples were obtained by venipuncture. Each sample was mixed with 0·1 ml. of 1 per cent sodium heparin in isotonic saline and the plasma separated by centrifugation at 3000 r.p.m. for 10 min. Plasma samples so obtained were tested within one hour.

Before testing, recipient animals were " blued " by the intravenous injection of 5 mg./kg. of T-1824 (Evans Blue) made up as a 1·5 per cent solution in isotonic saline. T-1824 is known to bind securely to the plasma albumin (Rawson, 1943). When test samples were thereafter injected into the recipient's skin, the degree of dyeing at the injection site provided an index of increased capillary permeability.

Duplicate intradermal injections of non-autologous and autologous plasma samples were made on the clipped back of the recipient with tuberculin syringes fitted with 26 S.W.G.

needles. The volume injected was 0·1 ml. Test sites were examined 30 min. after injection; the diameter of the blued area was measured, and the intensity of blueing graded from 1 to 4.

*Puppies*

Plasma obtained from puppies was tested in adult recipients. Both donors and recipients were mongrel animals, unselected as to breed or sex, from the animal colony at McGill University. None of the animals had previously been exposed to dog plasma or serum.

Two litters of puppies were followed at weekly intervals from the 10th day after birth until their plasmas produced consistently positive reactions in adult recipients. For this testing of puppy plasma, one adult recipient was used per litter per week. Blood samples (3·0 ml.) were taken from each puppy by cardiac puncture. Each sample was heparinized with 0·1 ml. of 5 per cent sodium heparin in isotonic saline, and the plasma harvested after centrifugation. Plasma samples were left unpooled. An aliquot from each sample was serially diluted with isotonic saline. The dilutions were tested immediately, and the highest dilution at which activity was retained was noted. This procedure, by comparison with adult controls, provided a rough index of non-autologous plasma activity in those cases where neat plasma produced pronounced reactions. The procedure for intradermal injection and reading of injection sites has been described.

As controls, one adult donor animal was assigned to the first litter (litter A) and two adult donor animals to the second litter (litter B). These control animals were bled and their plasmas tested concurrently with the puppies, on the same recipients.

Undiluted plasmas from a third litter, taken 12 hr. after birth, were tested on one occasion.

## RESULTS

*Inbred dogs.*—The results are presented in Table I.

In expt. 1, the responses were tested of one recipient Basenji to plasma from animals of other breeds, and to plasma from animals of his own breed. It was found that the non-autologous plasma effect occurred in these pure-bred animals, that it was pronounced, and that it did not appear to diminish as the genetic relationship between donor and recipient became closer.

Closely related Basenjis were also examined in expt. 2, and the results were similar to those of the first experiment. In expt. 2, 3-fold serial dilutions of plasma were also tested; this method provided a better index of plasma activity than did simple testing of undiluted plasma. The highest dilution which was still active was 1/27 for every non-autologous plasma, indicating that they all possessed approximately the same activity. Control testing of diluted autologous plasma showed no evidence of the appearance of the permeability factor described in guinea-pig plasma by Miles and Wilhelm (1955). In expt. 3, all possible donor-recipient pairs were tested among 5 Basenji litter mates (animals F, G, H, I and J of the pedigree). The non-autologous plasma effect occurred in every combination. The results with Basenji F as recipient are shown in the table; when his siblings were used as recipients, they showed just as pronounced reactions.

In expt. 4, the litter mates and sires from an inbred strain of Springer Spaniels were examined. Although the non-autologous plasma effect appeared to be more variable here, it was still present in every pair. Much of the variation was probably due to the difficulty in assessing the intensity of dyeing at the injection sites, for these animals had naturally dark skins.

Many of the closely related pairs were typed for erythrocyte agglutinogens using the system developed by Young and his associates (1951). There was no correlation whatever between blood group compatibility and reactivity to non-autologous plasma.

TABLE.—*Cutaneous Responses Among Inbred Dogs to Intradermal Injection of 0·1 ml. of Autologous or Non-autologous Plasma*

| Expt. No. | Recipient | Donor | Response: Diameter (mm.) | Response: Intensity |
|---|---|---|---|---|
| 1 | Basenji S | Self | 0 | — |
| | | Beagle | 13·0 | 3 |
| | | Cocker | 11·5 | 4 |
| | | Springer | 14·5 | 4 |
| | | Basenji C | 12·3 | 4 |
| | | E | 13·5 | 4 |
| | | K | 12·0 | 4 |
| | | Q | 12 8 | 4 |
| 2 | Basenji U | Self | 0 | — |
| | | Basenji C | 11·0 | 4 |
| | | Q | 10·8 | 3 |
| | | V | 12·8 | 4 |
| | Basenji V | Self | 0 | — |
| | | Basenji U | 13·8 | 4 |
| 3 | Basenji F | Self | 0 | — |
| | | Basenji G | 13·0 | 4 |
| | | H | 12·8 | 4 |
| | | I | 14·0 | 4 |
| | | J | 13·0 | 4 |
| 4 | Springer O | Self | 0 | — |
| | | Springer P | 11·8 | 3 |
| | | Q | 7·0 | 1 |
| | | R | 11·8 | 3 |
| | | S | 12·0 | 2 |
| | | T | 9·8 | 2 |
| | | U | 9·5 | 2 |
| | | M | 12·0 | 3 |
| | | L | 13·0 | 4 |
| | Springer Q | Self | 0 | — |
| | | Springer O | 9·8 | 1 |
| | | R | 10·3 | 3 |

*Puppies.*—Undiluted puppy plasma tested on the 10th, 17th and 24th post-natal days uniformly produced either no response, or weak responses, in adult animals. From the 31st day on, however, the activity rapidly increased to the adult level. The results obtained from litter B are shown in Fig. 2. Litter A followed a similar pattern, although plasmas from litter A reached a somewhat higher level of activity by the 8th post-natal week. The detailed results from litter A will be presented in a later communication in conjunction with electrophoretic studies on the plasmas from this litter.

Litter A was weaned during the 4th post-natal week. Since the increase in plasma activity to the adult level occurred during this period, it seemed interesting to see whether weaning influenced the time course of the increase. Weaning of litter B was therefore withheld for the duration of the experiment. Evidently this did not prevent the development of adult activity.

Undiluted plasma samples taken from a litter of 8 puppies 12 hr. after birth produced reactions in an adult recipient comparable to those produced by adult

plasma. The mean diameter of the blued areas was 9·8 ± 1·4 mm. (S.D.) with an average intensity of 3½.

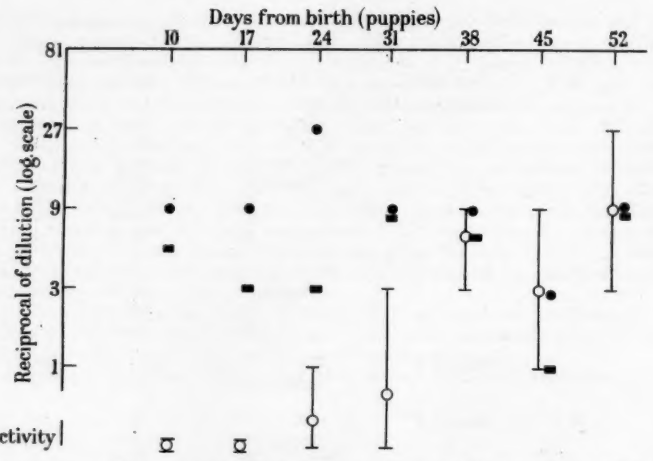



FIG. 2.—Titres of non-autologous activity in plasma from puppies and from adult dogs. ⊼: mean titre, with full range, of litter of 5 puppies; ● ■: titres of two adult animals tested concurrently on same recipient.

## DISCUSSION

The results obtained with inbred animals clearly demonstrate that the selective response of skin to autologous and non-autologous plasma is not a phenomenon restricted to mongrel dogs. Among 38 donor-recipient pairs of inbred adult animals of standard pure breeds, non-autologous plasma produced a response in every instance; autologous plasma never produced detectable reactions in these animals. Further, the responses to non-autologous plasma among inbred animals seem to be just as pronounced as those among mongrel dogs. Bliss and Stewart (1957), using 3-fold serial dilutions of donor plasma, found that activity among mongrel dogs was usually retained to a 1/9 dilution. In expt. 2, where a similar dilution method was used to assess the activity of donor plasma, the activity was retained at a dilution of 1/27 among closely related Basenjis.

The most closely related animals we studied (Basenjis U and V of expt. 2, Springers O–U of expt. 4) were sufficiently inbred to be heterozygous for an average of only 10 per cent of the loci at which heterozygosity existed in the original *pure-bred* population. Since these animals showed the non-autologous plasma effect without exception, the degree of individual specificity of dog plasma must be extreme.

This evidence eliminates gross ontogenetic disparity among dogs as an explanation for the non-autologous plasma effect. The phenomenon therefore remains unexplained. In two ways it resembles an antigen-antibody reaction: the response occurs rapidly after exposure, usually within 5 min. after injection, and a release of endogenous histamine is almost certainly involved. We feel that these similarities,

and the elimination of large ontogenetic disparity as an essential factor in the phenomenon, justify the tentative hypothesis that the non-autologous plasma effect is a manifestation of an antigen-antibody relationship between skin and plasma, with the antibodies occurring naturally. If this is so, however, the number of antigenic types must be relatively large to account for the degree of specificity encountered even with closely related animals.

The inbred animals we studied had never been previously exposed to donor plasma ; but it is possible, of course, that they had been isoimmunized by exposure to pooled dog serum given to protect against distemper, or by exposure to cellular material contained in the anti-hepatitis vaccine. With this in mind, we have recently been able to test a number of pure-bred Springer Spaniels which had never received dog serum or cellular material of any kind. Twenty donor-recipient pairs have been tested among litter mates, and 22 pairs among less closely related animals. The non-autologous plasma effect occurred in every pair. We consider that these results indicate that the possibility of isoimmunization need not be a serious qualification to the interpretation of the present study.

Plasma taken from puppies during the early weeks of life is ineffective in producing the typical response to non-autologous plasma. This is particularly apparent in view of the fact that among more than 200 donor-recipient pairs of mongrel and pure-bred adult dogs we have encountered no instance in which non-autologous plasma has failed to produce the expected response ; among 33 samples of plasma obtained from 11 puppies during the 2nd, 3rd and 4th post-natal weeks, however, 20 samples failed to produce any detectable response in adult recipients, and the remainder produced only weak responses. It should be noted, however, that plasma samples taken from animals 12 hr. after birth were quite active, suggesting a placental or colostral transfer of some factor necessary for the non-autologous plasma effect. The stimulus which causes plasma activity to rise to the adult level after the 4th post-natal week remains unknown. Although in litter A the rise to adult level was concurrent with weaning, litter B showed a similar rise while unweaned.

The results obtained with plasma from puppies are at least partly interpretable on the basis of an antigen-antibody hypothesis. Young members of many species are known to be deficient in natural antibodies (Landsteiner, 1945). We do not submit this as evidence that the antibody-like member of the system lies in the plasma, however, for in at least some instances young animals are antigenically immature as well (*e.g.*, Formaggio, 1951).

We must emphasize that we postulate an antigen-antibody system only as a working hypothesis. Another possibility, perhaps less plausible but more intriguing, is that the non-autologous plasma effect is a manifestation of the unknown mechanism whereby an animal is able to recognize all mature homologous tissues, excepting those from a uniovular twin, as being " foreign ". That this mechanism exists is required by the immunological explanation for homograft rejection.

We are continuing to inbreed the Basenji line to reduce still further the probability of genetic heterogeneity, and also are attempting to extend our observations to other species. Miles and Wilhelm (1955), Bliss and Stewart (1957), and Battisto (1957) have all observed cutaneous reactions to homologous non-autologous plasma or serum in a small proportion of guinea-pigs, and it is possible that with more refined techniques these animals may provide an opportunity for a more definitive genetic study of the phenomenon. Bliss and Stewart (1957) have also

observed cutaneous reactions to non-autologous plasma apparently unrelated to major blood group incompatibility in human subjects, and a larger study of human subjects is in progress.

SUMMARY

Mongrel dogs uniformly react with a marked increase in local capillary permeability on first exposure to intradermally injected plasma from other (adult) dogs, but do not so react to a similar injection of their own plasma. This phenomenon has been investigated in partially inbred dogs of standard pure breeds. Among 38 pairs of such animals, many of whom were closely related genetically, all showed responses to non-autologous plasma, but none to autologous plasma. The degree of individual specificity exhibited by dog plasma is therefore extreme.

Plasma from mongrel puppies, in contrast, often failed to produce typical responses.

The implications of these findings are discussed.

We are indebted to Dr. A. S. V. Burgen and to Dr. F. C. MacIntosh for their advice and encouragement during this investigation. The work was generously supported by Eli Lilly & Co., the National Research Council of Canada, and National Health Grants.

We are particularly grateful to Baron A. P. de Mengden of Montreal for providing us with some non-immunized pure-bred Springer Spaniels.

## REFERENCES

Battisto, J. R.—(1957) *Fed. Proc.*, **16**, 406.
Bliss, J. Q. and Stewart, P. B.—(1957) *Canad. med. Ass. J.*, **76**, 847.
Formaggio, T. G.—(1951) *Proc. Soc. exp. Biol.*, N.Y., **76**, 554.
Freeman, N. E. and Schecter, A. E.—(1942) *Science*, **96**, 39.
Lake, B. J., Simmonds, W. J. and Steinbeck, A. W.—(1953) *Aust. J. exp. Biol. med. Sci.*, **31**, 55.
Landsteiner, K.—(1945) 'The Specificity of Serological Reactions', ed. 2. Harvard Univ. Press, p. 131.
MacKay, I. R., Larkin, L. and Burnet, F. M.—(1957) *Lancet*, ii, 122.
Miles, A. A. and Wilhelm, D. L.—(1955) *Brit. J. exp. Path.*, **36**, 71.
Rawson, R. A.—(1943) *Amer. J. Physiol.*, **138**, 708.
Schachter, M. and Talesnik, J.—(1952) *J. Physiol.*, **118**, 258.
Young, L. E., Christian, R. M., Ervin, D. M., Davis, W., O'Brien, W. A., Swisher, S. N. and Yuile, C. L.—(1951) *Blood*, **6**, 291.

# THE EFFECTS OF ACUTE INFECTION AND ACUTE STARVATION ON SKELETAL DEVELOPMENT

## A Study of Young Rats

R. M. ACHESON* and M. N. MACINTYRE

*From the Moyne Institute of Preventive Medicine, University of Dublin and Department of Anatomy, Western Reserve University, Cleveland, Ohio*

Received for publication October 8, 1957

Analysis of the data collected during the earlier years of the Oxford Child Health Survey showed that pre-school children who lived in poor homes grew, and matured skeletally, more slowly than did their co-evals living in prosperous circumstances. By relating stature to level of skeletal maturity rather than to age, it was shown that the poor children suffered a certain amount of stunting, and the conclusion was drawn that, should the environmental difference between the two groups remain unchanged throughout the growing period, the poor children would become the smaller adults (Acheson and Hewitt, 1954). Subsequently Hewitt, Westropp and Acheson (1955), using the same material, showed that the common intercurrent infections of childhood also interfered with increase in stature, causing children who suffered such an illness to grow about $\frac{1}{4}$ in. less in that year of their lives than did others of the same age and sex who remained healthy during the same year. In this analysis, however, the retardation noted in the skeletal maturation of the hand and knee did not reach the limits of statistical significance. Thus it seemed that the sick children were maturing without interruption, and unless they were capable of accelerating their growth they would close their epiphyses (*i.e.* stop growing) $\frac{1}{4}$ in. smaller than they would have been had they not been ill. There was no suggestion of such an acceleration during the year following the illness, and it was concluded that such seemingly trivial incidents as attacks of acute bronchitis or measles in early life might possibly cause minor degrees of permanent stunting of adult stature.

In the field of animal experiment an accelerated gain in weight in kittens following release from daily exposure to chloroform was demonstrated over fifty years ago by Schapiro (1905) and a similar dramatic weight gain described in rats, which were fed after prolonged inanition by Osborne and Mendel (1916). Subsequently many studies of growth following chronic malnutrition have been conducted (Jackson, 1936; Quimby, 1951; Fábry and Hrůza, 1956), but to our knowledge no investigation has been carried out upon the effect of acute illness or starvation, nor have daily measurements of length been made.

In order to pursue further the long-term effect of acute trauma on skeletal development, animal experiments were planned to examine the validity of the following hypothesis: "An acute adverse experience, whether infection or

* Rockefeller Travelling Fellow in Medicine 1955–56 and Radcliffe Travelling Fellow, University College, Oxford.

starvation, has a general retrading effect upon the skeletal development of the growing mammal. The retardation in linear growth is greater than that in skeletal maturation, and as a consequence, a minor degree of permanent stunting may occur." The animal selected for the purpose was the rat because it is easy to handle and it undergoes the major part of its skeletal maturation after it has been born. In the latter respect it is similar to the human, in striking contrast to other laboratory animals.

Difficulty was experienced in keeping an animal sick for more than 48 hr., and yet being reasonably certain that it would completely recover. Therefore, two separate experiments were performed: in the first a group of animals were infected intraperitoneally with a Type I pneumococcus and later treated with penicillin and in the second another group was subjected to starvation for several days, thereby giving an opportunity for the study of a slightly longer period of acute environmental stress.

## MATERIAL AND METHOD

*Experiment I.*—Eight litters of albino rats of known age were used, each consisting of 8 animals. In one of these litters there were only 2 males, in three there were 3 males, in two there were 4 males and in two there were 5 males. In the three litters in which the sexes were divided evenly the rats were paired off by weight, so that there was a control for every animal to be infected. In the other five litters one control animal was used for two experimental ones, the matching again being done by weight, so that in all there were 29 male animals 12 of which were controls, and 35 females 15 of which were controls.

When the animals were 24 days old each had its weight, total length and tail length recorded while fully conscious (see Macintyre, Acheson and Oldham, 1958). The next day they were all radiographed under general anaesthesia, and the skeletal maturity status of each was assessed from the X-ray plates by the Oxford Method (Acheson, 1954, 1957; Macintyre, Acheson and Oldham, 1958). This method depends upon the recognition, on the radiogram, of shape changes in the parts of the maturing skeleton where osteogenesis is occurring. Such shape changes as are constant in all animals, regardless of sex, are described as maturity indicators, and the total number of indicators in any X-ray is known as the maturity score. Skeletal maturity scores for the rat ranged from a minimum of 22 at the age of 3 weeks to 100 at 16 weeks. Then the members of the experimental group (17 males and 20 females) were inoculated intraperitoneally with a Type I Pneumococcus. Each animal was inoculated with 0·1 ml. of a 1/10,000 saline dilution of a virulent strain which had just been cultured in blood broth for 4 hr. Eighteen hours later intramuscular injections of 5000 units of penicillin were given and repeated twice daily for 5 days.

Twenty animals (9 male and 11 female) failed to respond to penicillin in the first 24 hr., 4 died on the third day, 3 on the fourth day and one on the eleventh day. This animal, a male, after making a good initial response to the antibiotic, developed otitis media and despite strenuous treatment died aged 36 days. In the majority of these cases of premature death the control animal was killed. Eighteen animals, 9 from the experimental group and 9 controls, survived for the duration of the experiment.

Measurements of total and tail length, and of weight were recorded at the age of 26 days and daily thereafter to 40 days of age. They were repeated at the ages of 44, 54, 64, 94 and 114 days, when the animals were killed. X-rays were taken at the ages of 33, 39, 42, 55, 65, 95, and 114 days.

After death the animals were all dissected; from the left side of the body the third metacarpal, radius, ulna, humerus, femur, tibia and third metatarsal were cleaned and the length measured with callipers accurate to 0·2 mm. The final value for each bone was the average of three independent readings (Acheson and Macintyre, 1958). The same bones from the right side, together with sections of the tail were decalcified and sectioned for histological examination.

*Experiment II.*—Five litters were used, each again consisted of 8 animals, but on this occasion the sexes were equally divided (4 males and 4 females). The animals were sexed, the males in each litter numbered 1–4, and the females 5–8; all animals were earmarked for

purposes of identification. Then one male and one female from each litter were selected to act as controls ; no account was taken of size when this selection was made.

In order to obtain a good baseline the same daily measurements as had been used in the first experiment were instituted at the age of 21 days, 5 days before the animals were starved ; these measurements were continued until the age of 35 days. They were then repeated at the age of 40 days, and on every tenth day thereafter until the age of 70 days, then again at 90 and 110 days when the animals were killed. X-rays were taken at the ages of 32, 42, 52, 63 and 110 days.

All food was removed from the cages of the experimental animals after they had been measured on the morning of the 27th day (26 days old) and they were left with a diet of water for 48 hr. On the 29th day these animals were allowed to feed normally for 24 hr. before having their food removed for a further 48 hr. (the 30th and 31st days). *Ad libitum* feeding was reintroduced on the 32nd day and continued until the end of the experiment. Two males died before the experiment started, 4 females failed to recover from the period of starvation, and one male died under anaesthetic aged 60 days.

After the remaining 33 animals had been killed, some bones from the left side were measured, and others from the right prepared for histological examination in the same manner as has been described for Experiment I.

*Assessment of results.*—In order to judge the immediate effect of the infection or starvation upon the experimental animals the following procedure was adopted. The difference $d$ between the total length of each experimental animal and its control was calculated every time they were measured. If the value for the experimental animal was the smaller it was designated by a minus sign, and if larger by a plus. Then, since a difference of say 2 mm. between 25 day-old rats is a larger proportion of their total growth than is the same difference between two mature rats, the value $d$ was expressed as a percentage of the mean length of all the control rats of the same age and sex. Finally the results for males and females were combined by estimating the weighted mean of the percentage values for each.

$$\text{Per cent deviation length} = \frac{\text{Length of experimental animal—Length of control}}{\text{Mean length of all controls of same age and sex}} \times 100$$

$$\text{Per cent deviation of males and females combined} = \frac{(\text{Per cent male deviation} \times \text{No. of males}) + (\text{per cent female deviation} \times \text{No. of females})}{\text{No. of males} + \text{No. of females}}$$

The same procedure was then followed with respect to tail length, weight and skeletal maturity Finally, the difference was established between the lengths of the dissected third metacarpal and third metatarsal of each experimental animal and those of their controls.

## RESULTS

The results of Experiment I are shown in Fig. 1. The horizontal line represents a mean difference of zero between the experimental animals and their controls ; all points below this line indicate that, on average, the experimental animals were smaller than their controls, and points above indicate the reverse. For the sake of simplicity tail length has been omitted from the graph because it follows the pattern of total length very closely. It will be seen that all the three facets of physical development studied are slowed in the sick animals, weight being the most profoundly affected and skeletal maturation the least. By the fifth post-infection day, however, the experimental group had started to gain weight on the controls ; this gain continued, albeit erratically to begin with, until the 15th post-infection day, at which time the rate of gain flattened out. Growth took longer to recover, and it was not until the seventh post-infection day that this process showed any sign of making good its losses ; in spite of the fact that clinically

the rats gave the appearance of having been cured by the third post-infection day. By the time the animals were killed the total length curve had almost returned to the level from which it had started, but the experimental animals were still 1·8 per cent lighter than their controls. However, these differences were not statistically significant, although the deviations throughout the greater part of the experiment had been highly significant.

The first gain for skeletal maturation is followed by a loss, but by the end of the experiment there is no difference between infected or uninfected animals in this respect either.

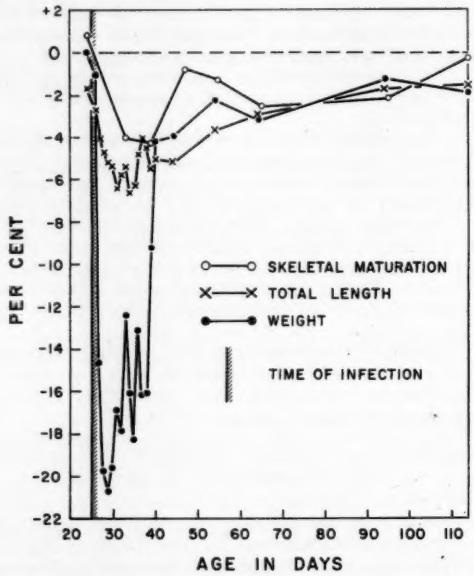



FIG. 1.—Differences between serial measurements of experimental and control animals in Experiment I, expressed as a percentage of the same measurements of all controls of the same age and sex.

Fig. 2 shows the results for the second experiment; it will be seen at a glance that the general pattern is the same, though the degree to which the animals were affected in the second experiment was much more profound. Between the first and second periods of starvation about 20 per cent of the weight loss is made up, while the growth process only just holds its own. Similarly after the second starvation period a dramatic weight gain (over 25 per cent of the total loss) is shown while the process of increasing in length is resumed, but at the same speed as that of the controls. This pattern is continued for 4 days after which the starved animals lose once more, although they are simultaneously gaining in weight. It is not until some 8–9 days after full feeding had been reinstituted that a definitive growth spurt began and the experimental animals started to catch up with their controls.

As in the first experiment skeletal maturation shows a loss to begin with and then, after a temporary gain, a second loss before returning to the same level as that from which it started.

Neither in linear growth, nor in weight increment, however, has the starved group succeeded in catching up with its controls, and the difference between the groups is highly significant (Table I).

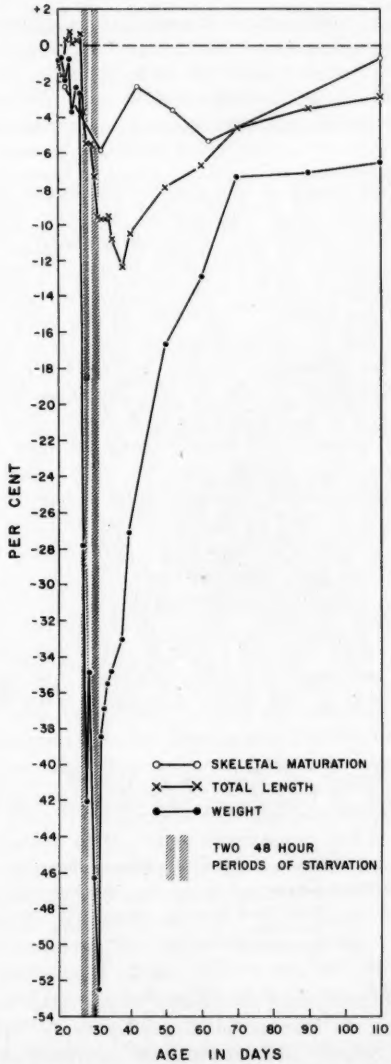



FIG. 2.—Differences between serial measurements made in Experiment II, expressed in the same way as was used in Experiment I (Fig. 1).

TABLE I.—*Mean Difference (and Standard Error of Mean) Between Experimental and Control Animals Aged* 110 *Days,* 79 *Days After Starving*

| | Males only (12) | | Females only (11) | | All animals (23) | |
|---|---|---|---|---|---|---|
| | Mean difference | Standard error of mean difference | Mean difference | Standard error of mean difference | Mean difference | Standard error of mean difference |
| Length (mm.) | −1·78 | ±0·43 | −0·41 | ±0·26 | −1·13 | ±0·29 |
| Weight (g.) | −37·8 | ±8·0 | −2·7 | ±4·8 | −21·0 | ±5·9 |
| Skeletal maturity (points)* | −1·67 | ±1·42 | +0·45 | ±1·06 | −0·65 | ±0·91 |

See Acheson, 1957, Macintyre *et al.*, 1958.

It is well known that many of the growth cartilages in the rat never ossify, and that others maintain minor degrees of activity until senescence. Thus, although the animals have achieved full sexual maturity by the age of 110 days they continue to grow slowly and therefore retain the ability to make up lost ground much later than would the human and, had the experiment been prolonged, might have made good the leeway. The bones of the metacarpus and metatarsus, however, are more similar to the primate in that their growth cartilage plates are ossified shortly after puberty. Becks, Asling, Collins, Simpson and Evans (1948) showed that ossification of the growth cartilage plate in the third metacarpal was well advanced in the majority of animals they studied by the 110th day of life, and that all growth had ceased about 20 days previously. Our histological studies confirmed this report, and we found that the third metatarsal tended to reach maturity at much the same time as the metacarpal. A disadvantage of using these bones is that by the time our experiments started (25th day of life) they has already undergone about 80 per cent of their entire growth, whereas in total length, the animals had completed less than 50 per cent of their ultimate growth. Nevertheless, since these bones followed the human pattern closely, we made a special study of them. The differences between experimental and control animals are shown in Table II where it will be seen that while the difference between experimental and control groups has been made good in Experiment I, the results of Experiment II are again consistent with some genuine effect of stress.

TABLE II.—*Mean Difference in Millimetres Between Certain Bones of Animals Exposed to Infection or Starvation, and those of Litter-mate Controls of the Same Sex*

| Experiment | Bone | Mean difference | *P* |
|---|---|---|---|
| I | Third metacarpal | −·0625 | <·80 |
| I | „ metatarsal | −·0333 | <·90 |
| II | „ metacarpal | −·0571 | <·30 |
| II | „ metatarsal | −·1111 | <·20 |

*Sex differences.*—The survivors in the first experiment were too few to make a between-sex comparison. In the second experiment the males suffered a greater initial setback in every respect and showed more stunting at the end of the experiment than did the females. These sex differences are the same as those already

described in the rat by Jackson (1936). They are also similar to those postulated for the human in the Oxford Child Health Survey (Acheson and Hewitt, 1954; Hewitt, Westropp and Acheson, 1955; Acheson, Hewitt, Westropp and Macintyre, 1956), for not only is the retardation in the male greater, but its variability is also wider.

## DISCUSSION

It is always dangerous to argue from one mammalian family to another. Nevertheless, observations on the rat have borne out the first part of our hypothesis based upon observations of the human, for there can be no doubt that the stress, whether it was an acute infection, or acute starvation, did have a general retarding effect on skeletal development. Furthermore, this effect was more profound on linear growth than it was on skeletal maturation.

We can add, with equal certainty, that the accelerated weight gain which earlier workers (Schapiro, 1905; Osborne and Mendel, 1916; Jackson, 1937) have observed to follow release from any retarding influence is accompanied by accelerated growth in length. Thus we confirm the work of Barnes, Sperling and McCay (1947) who based their conclusions on measurement of bones from X-rays of rats taken at intervals of some weeks. Jackson's (1936) suggestion that the early stages of weight gain are due to increased intestinal content may help to explain why growth in length lags behind weight gain for the first day or two. It does not explain why the experimental animals, having held their own for 4 days, lose ground a second time before making a definitive gain in length. This interesting observation cannot be explained on the present evidence and merits further investigation.

Taking into account our results and those reported in the literature, it would seem that the effect of stress on the final length of any bone depends upon three things, namely the amount of growth the bone has completed before the stress is introduced, the amount of growing time left after the stress has been removed, and the duration of the stress itself. The fact that the observed effect on the metacarpus and metatarsus was less than that on total length was probably due to the first of these considerations. In experiments where the stress is long drawn out, such as those of Barnes, Sperling and McCay (1947), the latter two factors must be related and supplementary, because a rat which has suffered malnutrition for a year or more is past its normal growing age when full feeding is reinstated.

Clarke and his colleagues (Clarke, Bassin and Smith, 1936; Clarke and Smith, 1938) concentrated their attention on bone length and composition and showed that, while mild dietary deprivation for a short period of time could result in over-compensation so that the experimental animals actually finished longer than their controls, prolongation of the stress invariably led to stunting, even though full diet was given for several mouths subsequently. Their work differed from ours in that the degree of under-feeding was so graded as to keep the body weight of the experimental animals constant, whereas we induced a profound loss of body weight in all the members of the experimental group. Thus, since our severe short-lived trauma wrought a similar effect to the milder, long-lasting stress used by the earlier workers, we feel justified in endorsing the conclusions of Clarke and his colleagues (loc. cit.) and of Jackson (1936) that the ultimate stunting effect is also dependent both upon the severity and duration of the stimulus which causes it.

More recently Fábry and Hrůza (1956) have exposed rats, over a period of 6 weeks, to intermittent starvation of a type similar to that used in the present study. Their animals were about 10 days older (to judge by reported weight) at the outset than ours, and weight alone was repeatedly assessed; yet it is of considerable interest that therapy with growth hormone during the recovery phase enabled the experimental group to grow at a greater rate than a control group which had also been starved, and thus, on the criterion of weight, to avoid being stunted. Quimby (1951), however, in a similar study, found that increase in skeletal length failed to make the same response as weight gain when an anterior pituitary extract was given during the recovery phase. The role of the anterior pituitary in interrupted growth is discussed more fully elsewhere (Acheson, and Macintyre, unpublished).

No clear grounds can be given for the different response of the two sexes, but it is of some interest that the rat also resembles the human in normal growth, for although in both the female tends to be smaller, her skeletal maturation proceeds more quickly than that of the male (Macintyre, Acheson and Oldham, 1958).

In conclusion, our findings have supported our hypothesis that an acute adverse experience such as septicaemia or starvation has a general retarding effect on the growing mammal, and that the retardation of linear growth is greater than that of skeletal maturation. There was, however, pronounced compensation during recovery so that a 48-hour illness, had no perceptible permanent effect upon the skeleton, but intermittent acute starvation lasting over 5 days did appear to cause minimal stunting of bones incapable of growth into old age. It is not unreasonable to suppose that some of the inter-current illnesses of childhood may also cause minor degrees of permanent stunting in the human. This could only be confirmed by making repeated careful measurements of a small group of children at monthly intervals for several years and correlating the results with detailed health records.

## SUMMARY

Two experiments are described which were designed to test the hypothesis that an acute adversity such as an intercurrent infection or a period of total starvation so interferes with skeletal development as to induce minor degrees of permanent stunting.

Including controls a total of 104 3-week-old rats were used. It was found that both pneumococcal septicaemia, treated by penicillin, and also intermittent starvation lasting over 5 days, had a profound retarding effect upon increase in length, weight and skeletal maturity. The animals that suffered the infection which made them sick for about 48 hours had caught up with their controls by the age of 16 weeks, while those which had been starved showed residual stunting. This was true both of total length, which normally would have continued to increase several months after the experiment was complete and of bones of the metacarpus and metatarsus which had already ceased growing. The retardation experienced by the male was, in every respect, more severe than that experienced by the female.

It is concluded that a relatively short acute traumatic episode can cause permanent stunting of the skeleton in the rat and that the hypothesis is borne out for this animal. More research is necessary to ascertain whether similar stunting occurs in the human.

We should like to express our gratitude to Dr. Normand L. Hoerr for his interest and encouragement, to Mrs. Eleanor Oldham and Mr. William Golden for technical assistance and to Mr. David Hewitt for criticism and advice. Dr. Novelli, lately of the Department of Microbiology, Western Reserve University, kindly provided the Pneumococci. The research was supported in part by grants from the U.S. National Science Foundation, the Committee for Research for Problems in Sex, the U.S. National Academy of Sciences, the U.S. National Research Council and the Medical Research Council of Ireland.

## REFERENCES

ACHESON, R. M.—(1954) *J. Anat., Lond.*, **88**, 498.—(1957) *Clin. Orthop.*, **10**, 19.
*Idem* AND HEWITT, D.—(1954) *Brit. J. Prev. Social Med.*, **8**, 59.
*Idem*, HEWITT, D., WESTROPP, C. K. AND MACINTYRE, M. N.—(1956) *Amer. J. phys. Anthrop.*, **14**, 375.
*Idem* AND MACINTYRE, M. N.—(1958) *Irish J. Med. Sci.*, Series **6**, in the press.
BARNES, L. L., SPERLING, G. AND MCCAY, C. H.—(1947) *J. Geront.*, **2**, 240.
BECKS, H., ASLING, C. W., COLLINS, D. A., SIMPSON, M. AND EVANS, H. M.—(1948) *Anat. Rec.*, **100**, 577.
CLARKE, M. F., BASSIN, A. L. AND SMITH, A. H.—(1936) *Amer. J. Physiol.*, **115**, 556.
*Idem* AND SMITH, A. H.—(1938) *J. Nutr.*, **15**, 245.
FÁBRY, P. AND HRŮZA, Z.—(1956) *Physiologia Bohemoslovenica*, **5**, suppl. 10.
HEWITT, D., WESTROPP, C. K. AND ACHESON, R. M.—(1955) *Brit. J. Prev. Social Med.*, **9**, 179.
JACKSON, C. M.—(1936) *Amer. J. Anat.*, **58**, 179.—(1937) *Anat. Rec.*, **68**, 371.
MACINTYRE, M. N., ACHESON, R. M. AND OLDHAM, E. C.—(1958) *Ibid.* in the press.
OSBORNE, T. B. AND MENDEL, L. B.—(1916) *Amer. J. Physiol.*, **40**, 16.
QUIMBY, F. H.—(1951) *Ibid.*, **166**, 566.
SCHAPIRO, A.—(1905) *J. Physiol.*, **33**, xxxi.

# ANTIBODY SYNTHESIS BY HOMOTRANSPLANTED CELLS AND TISSUES. III. MECHANISMS OF ANTIBODY SYNTHESIS DURING THE SECONDARY RESPONSE IN THE RECIPIENT ANIMAL

A. B. STAVITSKY*

*From the Department of Microbiology, Western Reserve University, School of Medicine, Cleveland, Ohio, U.S.A.*

Received for publication October 12, 1957

PREVIOUS studies (Stavitsky, 1954*a*, 1957*a*, 1957*b*) were devoted to the development of methods for the study of antibody formation by homotransplantation of lymphoid cells derived from rats or rabbits during the secondary antibody response. These methods have been applied to the elucidation of the sites and general mechanisms of this response (Stavitsky, 1954, 1957*a*, 197*b*). The present experiments, which have been described briefly (Stavitsky, 1955*b*, 1956, 1957*c*) were designed to obtain more conclusive evidence regarding the mechanisms of this response in the recipients of cells from immunized donors. It had already been established (see Stavitsky, 1957*c* for review of evidence) that the antibody which appeared in the recipient was not due to active immunization by antigen associated with the transplanted cells and that there was not enough free antibody in the donor's cells to account for the amount of antibody produced in the host. Moreover, other studies with the homotransplantation system, employing isotopes (Taliaferro and Talmage, 1955; Stavitsky, 1956, 1957*c*) or comparisons of the relative amounts of donor cell material and amount of antibody produced in the recipient (Roberts and Dixon, 1955; Stavitsky, 1956, 1957*c*), suggested that a synthesis of antibody from free amino acids occurred in the recipient. In studies with actively immunized animals it has been shown that amino acids can serve as precursors of antibody (Schoenhermer, Ratner, Rittenberg and Heidelberger, 1952; Gros, Coursaget and Macheboeuf, 1952; Green and Anker, 1954) and that the injection of radioactive normal gamma globulin does not result in greater radioactivity of antibody than injection of radioactive serum albumin (Gros *et al.*, 1952). Although the latter results appear to eliminate the conversion of normal gamma globulin to antibody gamma globulin as the mechanism of this synthesis, it is likely that circulating gamma globulin cannot readily enter antibody synthesizing cells and thus, even if capable, could not participate in antibody synthesis. Green and Anker (1954) determined the relative specific radioactivities of the glycine in the metabolic pool, normal gamma globulin and antibody in actively immunized animals given glycine-$C^{14}$ and concluded that gamma glubulin was not a direct precursor of antibody and antibody was synthesized from free amino acids. They also postulated that an inactive precursor of antibody may be formed first and subsequently converted to serologically-active antibody.

Although the previous data were highly suggestive, it was felt that better evidence was required to establish that the synthesis of antibody, particularly the

* A part of this work was conducted during the tenure of an Established Investigatorship from he American Heart Association.

antibody which appears in the recipient of cells from immunized donors, represents *net synthesis* or *de novo* fabrication of an increased amount of antibody from amino acids rather than conversion of a pre-existing protein precursor to antibody. Evidence for the occurrence of a net synthesis of antibody and the mechanisms of this synthesis is presented in this paper. This problem also has been approached with an *in vitro* system with similar results and conclusions (Stavitsky, 1957*d*).

A secondary purpose of this study was to characterize the antibody-forming system in comparison with systems involved in other immune phenomena. Accordingly, attempts were made to produce antibody by transplantation of various types of soluble, subcellular and disrupted preparations derived from immunized cells. Some success with such preparations has been reported. Sterzl and Hrubesova (1956) recently described the appearance of antibody in neonatal rabbits which had been given various subcellular and nucleoprotein fractions from spleens of rabbits immunized with *Salm. paratyphi*. He interpreted these results as due to conversion to antibody of a precursor in these fractions. Jeter, Tremaine and Seebohm (1954) reported the transfer of delayed hypersensitivity to 2,4-dinitrochlorobenzene from sensitive to normal guinea-pigs with leucocyte extracts. Lawrence (1955) has transferred this type of hypersensitivity from sensitive to normal humans with disrupted leucocytes.

## MATERIALS AND METHODS

### *Animals*

The donor rabbits were male albinos which weighed 2·5–3 kilos. The recipient rabbits usually were also male albinos which weighed 0·5–1 kilo with the exception of those experiments in which 3–5-day-old rabbits were employed. They were fed Purina rabbit checkers and given water *ad libitum*. The donor and recipient rats were males of the Sprague-Dawley strain obtained from Holzmann Farms, Madison, Wisconsin. They were fed Purina checkers and given water *ad libitum*.

### *Antigens*

The " Purogenated " alum-precipitated *diphtheria toxoid* and *bovine gamma globulin* preparations were described in a previous paper (Stavitsky, 1957*a*). The author is grateful to Lederle Laboratories for furnishing the diphtheria toxoid. In the quantitative precipitin method (Kabat and Mayer, 1948) a purified diphtheria toxoid (K39/50 kindly furnished by Dr. Inga Scheibel) was employed. This preparation contained 2560 $L_f$ units per mg. protein N. For the preparation of the chicken anti-rabbit gamma globulin serum two preparations of *rabbit gamma globulin* were available. Preparation No. 1 was derived from an anti-pneumococcus Type I rabbit serum and was generously provided by Dr. Emil L. Smith of the University of Utah. The preparation and properties of this globulin were recently described (McFadden and Smith, 1955). The preparation contained 92 per cent of gamma component with an S value of 6·3 and 8 per cent of a component with a value of 8·3. Preparation No. 2 was made in our laboratory by the method of Nichol and Deutsch (1948) and according to its electrophetic pattern contained 98 per cent gamma-2 globulin. Approximately equivalent results were obtained with these preparations with an antiserum either to the homologous or heterologous protein used for immunization. *Ovalbumin* (thrice recrystallized —Armour Laboratories) was used for the production of rabbit antisera.

### *Antisera*

Chicken anti-rabbit gamma globulin sera were produced by the intravenous inoculation of 3·0 mg. of purified gamma globulin 4 times a week for 3 weeks followed by a bleeding 7 days after the last injection. Four antisera were prepared and tested by the quantitative precipitin procedure (Kabat and Mayer, 1948). Three of the antisera showed more than one precipitin peak, ranging from 2 to 3 clearly defined peaks, as well as a gradually rising curve which did not descend in the zone of antigen excess. The fourth antiserum possessed

the desired single peak and clear-cut antibody excess, equivalence and antigen excess zones. The last antiserum was used in the analysis of sera and tissue extracts for their rabbit gamma globulin content.

The ovalbumin aniserum was prepared by the intravenous injection of alum-precipitated ovalbumin into rabbits as described under the procedure for animals in Group A in the paper by Vaughan and Kabat (1953). This procedure yielded consistently potent antisera containing from 0·5–7·0 mg. specific antibody/ml of serum. The bovine gamma globulin antiserum was prepared by the intravenous injection of alum-precipitated ovalbumin by the same procedure as described above for ovalbumin. Rat and rabbit antisera to diphtheria toxoid were prepared by two injections of 16–20 $L_f$ units alum-precipitated toxoid separated by an interval of at least 28 days and bleeding 7–8 days after the second injection.

*Antibody assays*

The *quantitative precipitin procedure* was carried out essentially as described by Kabat and Mayer (1948) with the exception that the method of Lowry, Rosebrough, Lewis and Randall (1951) was employed for the measurement of protein. Purified rabbit gamma globulin (McFadden and Smith, 1955) was used to standardize this method. As applied to the measurement of rabbit gamma globulin in cell or tissue extracts considerable difficulty was experienced in obtaining the same standard curve with the standard globulin and the standard antiserum. Therefore, a standard curve was run each time the analyses were performed. Eight per cent NaCl was employed as diluent in these reactions in order to obtain maximal precipitation (Goodman, Wolfe, and Norton, 1951). Occasionally the hemagglutination reaction (Boyden, 1951) as modified in this laboratory (Stavitsky, 1954*b*) was used for the detection of small amounts of antibody.

*Isolation and analysis of proteins and antibody of cells, tissues and sera*

Lymphoid cells usually were isolated by teasing the spleen or lymph nodes with the prongs at the cut edge of 60-mesh stainless steel wire mesh. One set of prongs was employed to anchor the tissue and the other to comb out the cells. The cells were suspended in Earle's saline (1948) containing 0·125 per cent gelatin. However, when the cells were to be analyzed for their protein content the gelatin was omitted from the suspending medium.

Two types of extracts were employed for analysis of cellular protein content. The cells were frozen and thawed 6 times, alternately at −79° and 37°. The debris was spun out at 5000 r.p.m. for 30 min. In the alternate procedure the cells were suspended in 5 ml. of saline and exposed to sonication in a 9000 kilocycle Raytheon oscillator for 10 min. under a hydrogen atmosphere. Hydrogen was employed because of the indication from the studies of Grabar, Seligman and Bernard (1955) that leukocyte antigens might be destroyed by oxidation due to cavitation and denaturation in the oscillator. The temperature of the chamber of the oscillator was kept at about 5° by circulating water at 3° through a chamber jacketing the cylinder in which the cells were sonicated.

An equal volume of 5 per cent trichloracetic acid (TCA) was added to both types of preparations to precipitate the protein. When these procedures were employed on aliquots of the same cellular or tissue preparation it was found that the sonic procedure consistently resulted in the finding of a higher protein content, whereas the freezing and thawing procedure resulted in the finding of more gamma globulin. Therefore, the sonic procedure was employed for the protein analyses and the freezing and thawing method for the gamma globulin analyses

Antibody or gamma globulin was isolated from the serum by precipitation with ammonium sulfate (50 per cent concentration) or by alcohol (Nichol and Deutsch, 1948). In the latter procedure only the first precipitation to yield precipitate A was employed. The precipitate prepared by salting-out was dialyzed in the cold for three days against frequent changes of saline. The alcohol precipitate was not dialyzed but resuspended in saline after the supernatant was carefully decanted and the inside and mouth of the tube were wiped with facial tissue wrapped around a wooden applicator stick.

The precipitates resulting from these procedures were analyzed for antibody and gamma globulin by the procedures described.

*Isotope experiments*

$S^{35}$ L-methionine (16·3 mC/mg.) and $S^{35}$ yeast protein hydrolysate were obtained from Abbott Laboratories. The hydrolysate contained radioactivity in the following percentages

of labelled compounds as determined by paper chromatography: 30–50 per cent L-cystine, 20–40 per cent L-methionine and 5–15 per cent glutathione.

For determinations of radioactivity the globulins of the tissue homogenate were precipitated with alcohol as precipitate A of the Nichol-Deutsch method (Boyden, 1951). Radioactive substances, presumably complement, which might be adsorbed non-specifically on the antigen-antibody aggregate and give a false high value, were removed by a heterologous-albumin-anti-egg albumin antibody system, essentially as described by Keston and Katchen (1956). This procedure did not remove any of the antibody to diphtheria toxoid or bovine gamma globulin. Diphtheria antitoxin and anti-bovine gamma globulin antibodies were precipitated by a concentration of purified toxoid (K39/50) and bovine gamma globulin respectively which completely precipitated antibody at the equivalence point. The precipitates were washed twice in cold saline containing 10 micro g. of L-methionine per 100 ml. and dialyzed in the cold for 3 days against two changes of 1·5 liters of saline The precipitate was collected by centrifugation and redissolved in saline. One hundred to 750 micro g. of protein were pipetted into a steel planchet, dried and radioactivity determined with a Nuclear flow counter taking 3200–6400 counts. The amount of antibody in the precipitate was calculated from the equivalence ratio of antibody to antigen so that the radioactivity of the antigen-antibody precipitate could be recalculated in terms of the activity of antibody itself.

Occasionally, not enough antibody was produced in the recipient animal to isolate by direct precipitation with antigen. Antibody was co-precipitated by the addition of optimal ratios of antigen and antibody after non-specifically absorbable radiaoctive materials were removed as described before by the addition of a heterologous antigen-antibody system.

As a check on the ability of these methods to eliminate non-specific radioactivity, it was found that toxoid-antitoxin precipitates prepared in the presence of serum from non-immunized animals to which $S^{35}$ methionine was added contained very little radioactivity (background or $\frac{1}{3}$ greater.)

*Fractionation and disruption of lymphoid cells or tissues*

*Nuclear fractions* were prepared by the procedures of Schneider and Hogeboom (1950) utilizing 0·25 M sucrose as the medium. The *supernatant* contained mitochondria, microsomes and the soluble fraction. The nuclear fraction often was contaminated with morphologically-intact cells. However, most of these cells took up trypan blue which is an indication of increased cellular permeability as a result of cell damage. *Blendorized extracts* were prepared by use of a Waring blendor for varying lengths of time. The temperature within the blendor chamber was maintained at about 5° by circulating water chilled to 3° through a jacket outside the chamber. The tissues blendorized for 30 sec. still showed many intact cells, whereas treatment for three min. or more resulted in preparations devoid of recognizable cells. *Frozen and thawed extracts* were made by alternately freezing the cells at −79° in a dry ice-methyl cellusolve bath and rapidly thawing them by immersion in a 37° water bath. These extracts were stringy and viscous and undoubtedly contained a high concentration of highly polymerized nucleic acids and nucleoprotein.

*General aspects of experiments*

The experiments were performed according to the general methods described in previous papers (Stavitsky, 1954*a*, 1957*a*, 1957*b*, 1957*d*). Rabbits were injected intravenously when spleens were to be used as a source of cells or into the hind foot pads when the popliteal lymph nodes were employed. When the mesenteric lymph nodes were to be utilized the antigen was injected directly into these nodes. Rats always were inoculated intraperitoneally and the spleens employed for the isolation of cells. Rabbits and rats usually were inoculated on days 1 and 30 or 60, and organs removed for transplantation on days 33 or 63. Rabbits were injected with 20 $L_f$ units of alum-precipitated diphtheria toxoid intravenously o into the hind foot pads and 4 $L_f$ into the mesenteric nodules. Rats were given 8–16 $L_f$ units of toxoid intraperitoneally. Rabbits were inoculated with 20 mg. of alum-precipitated bovine gamma globulin as the primary and secondary stimulus. All transplants were made intraperitoneally except for those summarized in Table II which were done intravenously. A small aliquot of each cell suspension was saved for analysis and the larger part transplanted. The recipient animals were untreated except for the recipients of disrupted and subcellular fractions of immunized cells described in Table IV. Since the latter experiments were based partially on the work of Sterzl and Hrubesova (1956) the recipients were neonatal rabbits of

3–5 days old as employed by them. Although the recipient animals usually were not treated to abolish their power to produce antibody against antigen in the donor cells, the antibody produced in them after transplantation appeared too rapidly (4–8 days) to be due to active immunization.

## EXPERIMENTAL

### *Incorporation of Radioactive Methionine into Antibody Produced in the Recipient*

Although it has been shown previously that amino acids can serve as precursors of antibody (Schoenheimer, *et al.*, 1952; Gros, *et al.*, 1952; Green and Anker, 1954), it had not been demonstrated in the homotransplantation system. Moreover, incorporation of amino acids into proteins provides some evidence for the occurrence of actual protein synthesis. Therefore, it was desirable to carry out such experiments. Since the conclusion of the present study independent evidence that amino acids are incorporated into antibody in the recipient following homotransplantation has been published (Taliaferro and Talmage, 1955).

$S^{35}$ L-methionine was injected into recipients either with the donors' cells or at various times after injection of these cells. From the data in Table I it is clear that regardless of the time of injection this amino acid was incorporated into the antibody produced in the host animal. Moreover, the specific radioactivity of the methionine in this antibody closely approximated the specific activity of methionine in antibody produced by actively immunized animals given equal amounts of $S^{35}$ methionine shortly after the second injection of diphtheria toxoid. In an occasional experiment (No. 1) not enough antibody was synthesized in the recipient to be measurable but the antibody isolated by co-precipitation with known amounts of antigen and antibody was radioactive.

TABLE I.—*Incorporation of $S^{35}$ L-methionine into Antibody Produced in Recipient Animals*

| Expt. No. | Source of cells | Radioactivity to recipient | Recipient's serum* | |
|---|---|---|---|---|
| | | | Counts/min. | Per |
| 1 | Spleens of 4 rats given toxoid | 350 $\mu$C intraper. with cells | 1020 | mg. ppt. |
| 2 | Spleen of rabbit given bovine gamma globulin | 650 $\mu$C intraven. 6 days after cell transfer | 961 | mg. antibody. |
| 3 | Poplitial node of rabbit given diphtheria toxoid | 650 $\mu$C intraper. 6 days after cell transfer | 720 | mg. antibody. |
| 4 | Spleens of 2 rabbits given diphtheria toxoid | 560 $\mu$C intraper. 4 days after cell transfer | 686 | mg. antibody. |

* In Experiment 1 the animal was bled out 2 days after the administration of isotope. In the other experiments the animals were bled out 12–16 hours after injection of isotope.

### *Protein and Gamma Globulin Content of Transplanted Cells and Antibody Content of Recipient's Serum*

The results of the previous experiments suggested that antibody formation in the recipient involved incorporation of amino acids into antibody. However, neither these nor previous data provided strong evidence for the occurrence of an

actual synthesis of antibody by the transplanted cells in the recipient. Therefore, in this set of experiments the relationship between the total protein and gamma globulin content of these cells and the total antibody content of the recipient's serum was explored. Obviously, if antibody were derived from pre-existing cellular protein or gamma globulin the amounts of the latter two materials should exceed that of the antibody made from them. From a previous study (Stavitsky, 1954*a*) it was known that sometimes there was *less* protein in the transplanted splenic cells from immunized donors than antibody in the host animal. However, since the spleen contains many types of cells (erythrocytes, polymorphonuclear leukocytes, etc.) other than lymphoid cells, much if not most of the protein in this organ probably is associated with cells other than lymphoid elements. Since lymphoid cells constitute a major source of antibody (Harris and Harris, 1956) and lymph node cell suspensions consist predominantly of one cell type, lymphocytes (Harris and Harris, 1956; Chase, 1951; Roberts and Dixon, 1955; Stavitsky, 1957*a*, 1957*b*), it was preferable to utilize lymph nodes from immunized animals rather than spleens in these experiments.

Table II presents the results of a number of typical experiments. Experiment 5 confirms previous data (Stavitsky, 1954) in which a greater amount of protein was detected in an aliquot of the transplanted splenic cells than antibody in the recipient at the peak of the antibody response. However, even in this experiment there was not enough gamma globulin in the cells to account for the quantity of antibody produced. The remaining experiments show that there is a great discrepancy between the total protein and gamma globulin content of lymph node cells and the antibody content of the recipient of these cells. Increasing amounts of antibody gradually disappeared within the next week. Therefore, the total amount of antibody which was synthesized in the recipient probably was at least 2–3 times that indicated in the last column of this table.

TABLE II.—*Comparison of Total Protein and Gamma Globulin Content of Transplanted Cells with Total Antibody Content of Recipient Animals*

| Expt. No. | Source of donor cells | Transplanted cells: Protein (μg.) | Transplanted cells: Gamma globulin (μg.) | Recipient's serum: Total antibody* (μg.) |
|---|---|---|---|---|
| 5 | Spleen of 1 rabbit | 4,800 | <10 | 4,000 |
| 6 | Mesenteric nodes of 1 rabbit | — | 270 | 9,000 |
| 7 | Poplitial nodes of 1 rabbit. Nodes were analyzed and transplanted separately : | | | |
| | Left lymph node | 45 | 4 | 15,000 |
| | Right lymph node | 35 | 2 | 40,000 |

* At the height of antibody response in the recipients.

### *Conversion of TCA-insoluble Compounds to Antibody*

Although the previous experiments indicated that a net synthesis of antibody was occurring and this antibody could be derived from free amino acids, it was possible to a limited extent that other types of precursors could be converted to antibody. The data in Table II which showed a great discrepancy between the small amount of available cellular protein and the amount of antibody in the

recipient excluded the conversion of a substantial amount of pre-existing protein as the basis of the antibody response. However, it was possible that amino acids could be added to a polypeptide or protein to form some antibody. Accordingly, the potential TCA-insoluble tissue precursors were labelled and it was ascertained whether any of the label appeared in the antibody. It was essential that the TCA-soluble (amino acid-containing) fraction of tissue possess negligible radioactivity so that its contribution to the activity of antibody not be detectable. It was desirable to limit the total radioactivity of the TCA-insoluble fraction because cellular protein probably would be degraded in the recipient and radioactive mino acids derived from protein thereby be made available for the synthesis of antibody.

Since the tissue precursors were labelled with $S^{35}$ only their conversion to S-containing proteins such as rabbit antibody (Smith, McFadden, Stockell and Buettner Janusch, 1955) was detectable. It was calculated that if approximately 1 per cent of the TCA-insoluble labelled compounds were converted to antibody it would have been detected in these experiments.

The desired level and specificity of labelling could not be achieved by exposing the tissues to radioactive amino acids *in vitro* since this resulted in appreciable radioactivity in the T.CA-soluble fraction. The desired labelling was produced by injection of 500–1000 micro c of $S^{35}$ yeast protein hydrolysate into rabbits and about 250 micro c of hydrolysate into rats 16 hours before removal of tissues for transplantation.

Table III summarizes the results of these experiments. Transplants were done intravenously since it was shown in a previous study (Stavitsky, 1957*a*) that this resulted in a more rapid appearance of antibody in the recipient. It should also be noted that the proteins of the donor cells were labelled by injecting into the donor animal approximately as much radioactivity as was given to the recipient in the experiments in Table I. The data indicate that transplantation of immunized cells whose proteins were labelled did not result in appreciable labelling of the antibody formed within the recipient animals. Independent evidence resulting in a similar conclusion recently has been published (Taliaferro and Talmage, 1955).

Since it has been postulated that normal gamma globulin serves as a precursor of antibody gamma globulin (Pauling, 1940 ; Borsook, 1956), $S^{35}$-labelled normal rat and rabbit serum gamma globulin and albumin were injected into the recipients of cells from immunized rats and rabbits. The results as presented in Table III, Exp. 10, indicate that the antibody produced in these animals was labelled to the same extent whether gamma globulin or albumin was injected as the potential precursor.

### *Homotransplantation of Various Extracts and Fractions of Immunized Cells*

In these experiments we attempted to produce antibody by the transfer of various preparations derived from lymphoid cells during the secondary antibody response to diphtheria toxoid. These preparations were injected into neonatal rabbits as described by Sterzl and Hrubesova (1956). As shown in Table IV, antibody definitely was produced by transplantation of only those preparations (homogenates, nuclear fractions) which appeared to contain some morphologically-intact cells which did not take up trypan blue *in vitro*. Occasionally, as in Experiment 13 a small amount of antibody was produced by transfer of preparations

TABLE III.—*Failure of Conversion to Antibody of $S^{35}$-labelled TCA-insoluble Compounds*

| Expt. No. | Source of donor cells* | Analysis of transplanted material | | | | Recipient's serum | |
|---|---|---|---|---|---|---|---|
| | | TCA-insol. (counts/min.) | TCA-sol. (counts/min.) | Gamma globulin (counts/min.) | Alb. (counts/(min.) | Antibody ($\mu$g./ml.) | Radioact. antibody (counts/min./mg.) |
| 8 | Spleens of 4 rats; each given 130 $\mu$C $S^{35}$ hydrolysate 16 hours before removal of spleen | 10,500 | 310 | — | — | 17 | 0 |
| 9 | Spleens of 3 rats; each given 200 $\mu$C hydrolysate 16 hours before removal of spleen | 7,516 | 640 | — | — | 40 | 0 |
| 10 | Spleens of 4 rats | — | — | 380,000 | — | 20 | 86 |
| | | — | — | — | 437,000 | 32 | 79 |
| 11 | Lymph nodes of 2 rabbits; each given 500 $\mu$C hydrolysate 16 hours before removal of spleen | 7,360 | 1,752 | — | — | 76‡ | 0 |

* All donors were immunized with diphtheria toxoid.

† The figures given for gamma globulin and albumin represent the actual number of *total* counts per minutes of the protein injected intraperitoneally into the recipients. These proteins were derived from the sera of 5 rats which had been given 210 $\mu$C each of $S^{35}$ yeast hydrolysate and bled out 28 hours later. Globulin represented precipitate A by the Nichol-Deutsch procedure (1948) and the albumin the supernatant from this precipitate. Both proteins were lyophilized and reconstituted before use.

‡ Recipient was a neonatal rabbit.

which contained few if any intact cells. Since the neonatal rabbits did not manifest a primary antitoxin response to the injection of alum-precipitated diphtheria toxoid this minimal antibody response cannot be attributed to active immunization of the recipient by toxoid associated with the transferred cells.

## DISCUSSION

The data obtained in this study indicate that substantial antibody synthesis occurs in an animal after receipt of cells from immunized donors. The quantity of antibody produced is comparable to that formed in actively immunized animals (Steiner and Anker, 1956 ; Stavitsky, 1957*d*). This antibody apparently represents newly formed protein rather than the conversion of a pre-existing protein precursor to antibody (Holt, 1950) because there is only a very small amount of protein in the donor cells which after transplantation produce amounts of antibody up to 1000 times their original protein content. It was also found that not more than about 1 per cent of the antibody in the recipient could have been derived from pre-existing TCA-insoluble compounds. A similar conclusion has been drawn by Taliaferro and Talmage (1955). However, their transplants of labelled donor cells resulted in some labelling of the resulting antibody in contrast to the essentially negative results of the present study. This labelling may have been due to the fact that they injected 3 to 12 times more radioactivity into the donor animals than was employed in the present study. The radioactivity in the anti-

TABLE IV.—*Results of Transplantation of Various Fractions and Disrupted Preparations of Cells from Donors Immunized with Diphtheria Toxoid*

| Expt. No. | Source of preparations | Type of preparation | Recipient: Peak titer† | Recipient: Days after transfer |
|---|---|---|---|---|
| 12 | Spleens of 5 rats; 1/2 cells injected, 1/2 fractionated | Cells, untreated | 160 | 4 |
| | | Nuclear | — | — |
| | | Super.* | — | — |
| 13 | Spleens of 2 rabbits, cells fractionated | Nuclear | 640 | 7 |
| | | Super. | 40 | 11 |
| 14 | Popliteal nodes of 2 rabbits, cells fractionated | Nuclear | 160 | 7 |
| | | Super. | 0 | — |
| 15 | Spleens of 4 rats; 1/2 cells injected, 1/2 fractionated | Cells, untreated | 640 | 6 |
| | | Nuclear | 320 | 6 |
| | | Super. | 0 | — |
| 16 | Mesenteric nodes of 2 rabbits; 1/2 cells injected, 1/2 treated | Cells, untreated | 1280 | 4 |
| | | Cells, frozen and thawed | 0 | — |
| 17 | Popliteal nodes of 2 rabbits; 1/2 cells injected, 1/2 treated | Cells, untreated | 640 | 4 |
| | | Cells, 56° C. for 45 min. | 0 | — |
| 18 | Spleens of 2 rabbits; 1/2 cells injected, 1/2 blendorized | Cells, untreated | 320 | 6 |
| | | Cells, blendor 30 sec. | 320 | 6 |
| 19 | Spleens of 2 rabbits; cells divided in half for treatment | Cells, blendor 30 sec. | 320 | 4 |
| | | Cells, blendor 3 min. | 0 | — |

* Supernatant after removal of nuclear fraction.
† Reciprocal of hemagglutination titer.

body may, therefore, have been derived from the degradation of highly labelled cell proteins to labelled amino acids and *re*incorporation of these acids into antibody *or* from a highly labelled TCA-soluble (amino acid) cell fraction. Since no analysis of the radioactivity of the TCA-soluble and insoluble fractions of donor cells comparable to that in Table III was presented in the paper by Taliaferro and Talmage (1955) it is not possible to discriminate between these alternatives. That *re*incorporation does occur is shown by previous work (Gros, *et al.*, 1952) and current experiments (Table III, Exp. 10) in which labelled normal gamma globulin and albumin were injected into immunized animals or into recipients of immunized cells and the resulting antibody was isolated. Since the antibody was labelled equally whether it was derived from the amino acids of globulin or albumin it is most reasonable to assume that the activity of antibody was derived from reincorporation of labelled amino acids released by the degradation of the two serum proteins.

In contrast to the experiments of Taliaferro and Talmage (1955) just summarized, the experiments in Table III were done so as to minimize reincorporation of labelled amino acids derived from labelled cell proteins because the the protein of the cell fractions was of lower total and specific radioactivity than

in their experiments. Consequently it is possible that some direct conversion of cell protein to antibody was not detected (less than 1 per cent). However, this objection has been overcome in *in vitro* experiments in this laboratory (Stavitsky, 1957*d*). Under conditions rigidly controlled so as to minimize reincorporation and to attain high radioactivity in the potential TCA-insoluble precursors and negligible labelling in the cellular amino acids, the antibody synthesized was not radioactive. It could, therefore, be concluded that antibody was derived largely, if not exclusively, from free amino acids. Recent studies on the synthesis of adaptive enzymes in bacteria have reached essentially identical conclusions regarding the nature of the precursors of the newly synthesized enzymes (Hogness, Cohn and Monod, 1955).

The available data strongly suggest that the transplanted cells *per se* synthesize the antibody which appears in the recipient. Much of this evidence has been reviewed elsewhere (Stavitsky, 1957*c*). The amount of antibody produced by these cells and the extent and rate of amino acid incorporation into antibody in the recipient are of the same order of magnitude as if the cells had remained *in situ* (Taliaferro and Talmage, 1955; Schoenheimer *et al.*, 1952). Moreover, it appears that at least some of the transplanted cells were still actively engaged in antibody synthesis 6 days after injection since $S^{35}$ methionine given the recipient at this time was incorporated into antibody (Table I, Exp. 2 and 3). Since the transplanted cells apparently do not undergo extensive mitosis, although they may enlarge to some extent, in their new host (Dixon, Roberts and Weigle, 1957) the figures in Table II provide a good approximation of the relationship between the protein content of these cells and the amount of antibody they produce. Roberts and Dixon (1955) also concluded that these cells possess considerable synthetic ability by comparing the amount of antibody produced in the recipient with the *calculated* weight of the transferred lymphoid cells. However, the discrepancy between the amount of available cell material and the quantity of antibody formed in the host was not as great as was found in the present study by actual analysis of the donor cells and recipients' serum.

The small amounts of gamma globulin in lymphoid cell extracts may represent contamination with plasma gamma globulin. Abrams and Cohen (1949) found no component corresponding to gamma globulin in electrophoretic studies of extracts of human and calf lymphoid tissue. Harris, Moore and Farber, (1949) found no component with the sedimentation constant of gamma globulin in lymph node extracts after the injection of killed bacteria, although there was a component with the electrophoretic mobility of gamma globulin. Moreover, there was no increase upon immunization in the component with the same mobility as gamma globulin. Nor was there a material with the sedimentation constant of gamma globulin in normal lymphocytes.

Although the data effectively rule out the release of stored antibody or conversion to antibody of appreciable amounts of a pre-existing precursor as the basis of the secondary response, they do not elucidate the mechanisms whereby amino acids are condensed to form protein. The evidence does not exclude the presence in the cell of small amounts of a rapidly turning over precursor of antibody. Pauling (1940) and Borsook (1956) postulated that antibody results from the direct impression by the antigen of a complementary configuration upon normal gamma globulin or a polypeptide precursor. The available data do not exclude this hypothesis if it does not require the existence of large amounts of cellular

precursor and if the newly-synthesized antibody is released rapidly from the cell. Such a rapid release is in accord with studies which indicate that relatively little antibody is associated with antibody-producing cells compared to the amount of antibody in the lymph or blood (Stavitsky, unpublished) or in the medium *in vitro* (Stavitsky, 1955*a*). Recently, a different hypothesis has been proposed (Stavitsky, 1957*c*) in which the antigen serves both to induce the production of a cytoplasmic template for the synthesis of antibody as well as to trigger the secondary response in cells previously exposed to the antigen.

In this study and another one (Taliaferro and Talmage, 1955) the antibody associated with the transplanted cells was not recovered in the recipient if the cells were damaged before injection or if the transplant failed to cause antibody formation. Intracellular degradation of this antibody is consistent with the finding that lymphoid cells which were prevented from synthesizing antibody *in vitro* by cyanide or dinitrophenol nevertheless degraded some of the intracellular antibody (Stavitsky, unpublished ; Stavitsky, 1957*d*). The fact that this degradation occurs at pH values higher than the usual optimum for cathepsins and under aerobic conditions considered unfavourable for autolysis (Bradley, 1938) suggests that cathepsins are not operative in this situation. Since lymphoid cells can degrade antibody *in vivo* and *in vitro* the net synthesis of antibody which is demonstrable under these conditions actually represents the resultant of cellular synthesis and breakdown of antibody. Whether these anabolic and catabolic reactions are coupled is not known but recent studies suggest at least some relationship between these phenomena (Steinberg and Vaughan, 1956).

The absence of antibody formation after transfer of disrupted cells and cell fractions in this study and the production of antibody with such preparations in Sterzl's experiments (Sterzl and Hrubesova, 1956) cannot be explained at present. Sterzl employed splenic cells from rabbits primarily stimulated with *Salmonella*, whereas the present work employed cells from rabbits secondarily stimulated with toxoid. The significance of the low titers in his work is dubious because of the common presence of low titers of antibody to Gram-negative enteric organisms in normal animals (Wilson and Miles, 1955). Further study is required to clarify Sterzl's results, as well as to elucidate the mechanisms of the successful transfers of hypersensitivity in man and animals with disrupted cells or extracts. In view of the meagre information available on the mechanisms of delayed hypersensitivity it is premature even to speculate about the basis for the apparent difference between the phenomena of antibody production and the production of delayed hypersensitivity.

## SUMMARY

Radioactive methionine was incorporated into the antibody produced in the recipient after receipt of cells from immunized animals. The specific activity of the methionine in this antibody closely approximated the activity of methionine in antibody produced by actively immunized animals.

There was a great discrepancy between the total protein and gamma globulin content of lymphoid cells if immunized animals and the antibody content of the recipient of these cells. If antibody were derived from pre-existing cellular protein or gamma globulin the amounts of the latter two materials should have exceeded the amount of antibody made from them. The results suggest that the antibody in

the recipient was not derived from pre-existing complex molecules and, therefore, represented a net synthesis of new protein.

The potential TCA-insoluble tissue precursors of antibody in the transplanted cells were labelled with radioactive amino acids and it was ascertained whether the antibody produced in the recipient of these cells was labelled. It was found that the antibody formed in the recipient was not labelled under these circumstances and it was, therefore, concluded that the antibody was derived predominantly, if not exclusively, from free amino acids. Under appropriate experimental conditions it was possible, however, to demonstrate that breakdown of protein and reincorporation into antibody of the constituent amino acids could occur.

Homotransplantation of various extracts and fractions of cells from immunized donors led to antibody production only when preparations containing morphologically-intact cells were employed.

The author is very grateful to Anne D. Gardner and Frank C. Williams for excellent technical assistance.

The investigation was supported by a grant from the Life Insurance Medical Research Fund.

## REFERENCES

Abrams, A. and Cohen, P. O.—(1949) *J. biol. Chem.*, **177**, 439.
Borsook, H. J.—(1956) *J. cell comp. Physiol.*, Supplement 1, **47**, 35.
Boyden, S. V.—(1951) *J. exp. Med.*, **93**, 107.
Bradley, H. C.—(1938) *Physiol. Rev.*, **18**, 173.
Chase, M. W.—(1951) *Fed. Proc.*, **10**, 404.
Dixon, F. J., Roberts, J. C. and Weigle, W. O.—(1957) *Ibid.*, **16**, 649.
Earle, W. R.—(1948) *Proc. Soc. exp. Biol.*, **67**, 40.
Goodman, M., Wolfe, H. R. and Norton, S.—(1951) *J. Immunol.*, **66**, 225.
Grabar, P., Seligmann, M. and Bernard, J.—(1955) *Ann. Inst. Pasteur*, **88**, 548.
Green, H. and Anker, H. S.—(1954) *Biochim. biophys. acta*, **13**, 365.
Gros, P., Coursaget, J. and Macheboeuf, M.—(1952) *Bull. Soc. Chim. biol., Paris*, **34**, 1070.
Harris, T. N., Moore, D. H. and Farber, M.—(1949) *J. biol. Chem.*, **179**, 369.
*Idem* and Harris, S.—(1956) *Amer. J. Med.*, **20**, 114.
Hogness, D. S., Cohn, M. and Monod, J.—(1955) *Biochim. biophys. acta*, **16**, 99.
Holt, L. B.—(1950) *Brit. J. exp. Path.*, **31**, 233.
Jeter, W. S., Tremaine, M. M. and Seebohm, P. M.—(1954) *Proc. Soc. exp. Biol., N.Y.*, **86**, 251.
Kabat, E. A. and Mayer, M. M.—(1948) ' Experimental Immunochemistry '. Springfield, Illinois (Chas. C. Thomas).
Keston, A. S. and Katchen, B.—(1956) *J. Immunol.*, **76**, 253.
Lawrence, H. S.—(1955) *J. clin. Invest.*, **34**, 219.
Lowry, O. H., Rosebrough, N. J., Lewis, A. Farr, and Randall, R. J.—(1951) *J. biol. Chem.*, **193**, 265.
McFadden, M. L. and Smith, E. L.—(1955) *Ibid.*, **214**, 185.
Nichol, J. C. and Deutsch, H. F.—(1948) *J. Amer. chem. Soc.*, **70**, 80.
Pauling, L.—(1940) *Ibid.*, **62**, 2640.
Roberts, J. C. and Dixon, F. J.—(1955) *J. exp. Med.*, **102**, 379.
Schneider, W. C. and Hogeboom, G. H.—(1950) *J. biol. Chem.*, **183**, 123.
Schoenheimer, R., Ratner, S., Rittenberg, S. and Heidelberger, M.—(1952) *Ibid.*, **34**, 1070.

SMITH, E. L., MCFADDEN, M. L., STOCKELL, A. AND BUETTNER JANUSCH, V.—(1955) *Ibid.*, **214**, 197.

STAVITSKY, A. B.—(1954*a*) *J. Infect. Dis.*, **94**, 306.—(1954*b*) *J. Immunol.*, **72**, 360.—(1955*a*) *Ibid.*, **75**, 214.—(1955*b*) Symposium on Nutrition in Infections, *New York Acad. Sci. Art.*, 2, **63**, 211.—(1956) *Fed. Proc.*, **15**, 615.—(1957*a*) *J. Immunol.*, **79**, 187.—(1957*b*) *Ibid.*, **79**, 200.—(1957*c*) *Fed. Proc.*, **16**, 652.—(1957*d*) *Biochim. biophys. acta*, in press.

STEINBERG, D. AND VAUGHAN, M.—(1956) *Arch. Biochem.*, **65**, 93.

STEINER, D. F. AND ANKER, H. S.—(1956) *Proc. nat. Acad. Sci., Wash.*, **42**, 580.

STERZL, J. AND HRUBESOVA, M.—(1956) *Folia biol., B. Aires*, **2**, 21.

TALIAFERRO, W. H. AND TALMAGE, D. W.—(1955) *J. Infect. Dis.*, **97**, 88.

VAUGHAN, J. M. AND KABAT, E. A.—(1953) *J. exp. Med.*, **97**, 821.

WILSON, G. S. AND MILES, A. A.—(1955) ' Topley and Wilson's Principles of Bacteriology and Immunity ', Vol. II, p. 1234, Fourth Edition. Baltimore (Williams and Wilkins Co.).

# CHANGES ASSOCIATED WITH THE ACCUMULATION OF EXCESSIVE AMOUNTS OF IRON IN CERTAIN ORGANS OF THE RAT

L. GOLBERG AND J. P. SMITH

*From Benger Laboratories Ltd., Holmes Chapel, Cheshire, and the Department of Pathology, University of Manchester*

Received for publication October 17, 1957

THERAPY with parenteral iron has always been assumed to entail the risk that administration of excessive amounts of iron may lead to the development of haemochromatosis and other pathological sequelae. Direct evidence in support of this contention has never been forthcoming: indeed from observations on man and experimental animals it would appear highly unlikely that haemochromatosis can result from severe and protracted haemosiderosis (Golberg, 1957). Published data concerning the effects on animals of gross overloading with iron provide no evidence of an intrinsic toxic effect which may be exercised by prolonged tissue siderosis. The lesions which have been produced in animals treated parenterally with large amounts of colloidal iron have been the consequence of (i) instability of the colloid in plasma, (ii) liberation of free ionic iron in amounts which greatly exceed the serum iron-binding capacity, (iii) very high concentrations of undissociated iron complex in the blood, and especially in the glomeruli (Golberg, Smith and Martin, 1957).

The recent development of iron-dextran complexes which can be given to animals parenterally in huge quantities relative to the therapeutic dose has enabled the study of gross overloading with iron to be carried further. Some of the findings arising from work previously reported (Golberg *et al.*, 1957) suggested that there is a definite similarity between the changes in certain organs of the iron-loaded rat, notably the kidney and genital tract, and the pathological manifestations of vitamin E deficiency in this species (Martin and Moore, 1939). An effort has been made to establish the link between iron accumulation in tissues and the changes which are known to take place in those tissues when the whole animal, in this case the rat, is deprived of vitamin E over a long period.

## METHODS

*Original experiments*

Details of these experiments were provided in a previous publication (Golberg *et al.*, 1957). Altogether about 200 albino rats of both sexes were used. They were maintained on diet 41 (Bruce and Parkes, 1949, 1950) which contains 1 per cent of cod liver oil as the only added fat. The iron treatment consisted of intramuscular injections of an iron-dextran complex in doses of 25 and 75 mg. Fe/kg., given 2 and 3 times a week respectively to a total of 300–1650 mg. Fe/kg., *i.e.*, up to levels which correspond to about 100 times the total therapeutic dose. Control rats given equivalent amounts of similar low molecular dextran (without iron) were maintained on the same diet over the same period, *i.e.*, up to 60 weeks after the start of injections.

*Further experiments*

The new series (Table I) was carried out on similar lines to the earlier experiment, using diet 41 (Oxo) in which the cod liver oil is replaced by stabilised vitamins A + D. Its iron

content was found to be 0·044 per cent. Its tocopherol content was estimated by Dr. J. Green and found to be (in mg./100 g. diet): α 0·52; β 0·16; ζ 0·31; ε 0·35. One or other or both of two supplements were added: cod liver oil B.P. (soaked into the pellets to give a final concentration of 10 per cent w/w) and vitamin E (1 ml. of 10 per cent w/v solution of DL-α-tocopheryl acetate in oil given orally once a week). Iron to a total of 1650 mg. Fe/kg. was given by thrice weekly injections of 75 mg. Fe/kg. during the first 8 weeks of the experiment. The group symbols in the table denote the nature and duration of treatments, as well as the order in which they were given: Fe—iron-loading; E—vitamin E; O—cod liver oil. Treatments commencing on the seventeenth week and ending at 32 weeks are marked "17–32". Animals were killed at the end of the week in which their treatment stopped.

At autopsy the liver was weighed and stored at −20° until its vitamin A content was determined, using antimony trichloride. The change in renal non-protein nitrogen level was measured before and after incubation at 37° for 24 hr. Specimens of adipose tissue were collected from various sites, a part of each fixed in formalin and tested histochemically for the presence of peroxides (Hartmann and Glavind, 1949) and the remainder stored at −20° until estimation of the peroxide level could be carried out. For this purpose the method of Glavind, Granados, Hartmann and Dam (1949) was modified by the use of purified *n*-butanol as solvent.

*Histological procedures*

Sections were prepared from most organs in the body and were stained with haemalum and eosin, Perls' method for iron, Mallory's basic fuchsin, long Ziehl-Neelsen's stain for ceroid periodic acid-Schiff, acetyl Sudan black B and neutral red-tartrazine (Harris' haematoxylin, followed by 0·5 per cent neutral red at 60° for 30 min.; rinse well in water and differentiate with a saturated solution of tartrazine in cellosolve for 15–20 sec. Dehydrate, clear in xylene and mount in Xam. Renal ceroid stains red). Unstained sections mounted in Distrene 80 were examined for fluorescence in ultraviolet light. Significant findings reported here are seen in the kidneys, liver, uterus, testis, small intestine, lymph nodes and intra-abdominal and subcutaneous fat.

TABLE I.—*Experimental Details*

| Groups | Number of rats ♂ | Number of rats ♀ | Iron loading | Duration of treatment (weeks) Vitamin E | Duration of treatment (weeks) Cod liver oil |
|---|---|---|---|---|---|
| $FeE_4$; $FeE_8$; $FeE_{16}$; $FeE_{32}$ | 5 | 5 | + | 4, 8, 16, 32 | — |
| $FeE_{32}O_{17-32}$ | 2 | 3 | + | 32 | 16 (weeks 17–32) |
| $O_4$; $O_8$; $O_{16}$; $O_{32}$ | 4 | 4 | — | — | 4, 8, 16, 32 |
| $O_{32}E_{17-32}$ | 3 | 3 | — | 16 (weeks 17–32) | 32 |
| $FeO_4$; $FeO_8$; $FeO_{16}$; $FeO_{32}$ | 5 | 5 | + | — | 4, 8, 16, 32 |
| $FeO_{32}E_{17-32}$ | 2 | 3 | + | 16 (weeks 17–32) | 32 |
| $E_8$; $E_{16}$ | 1 | 1 | — | 8, 16 | — |
| $Fe_9$*; $Fe_{19}$*; $Fe_{32}$* | 9 | 4 | + | — | — |
| $Fe_{16}$ (continuing) | 2 | 2 | + | — | — |

* Original experiments, which continued up to 60 weeks.

## RESULTS

### *Original Experiments*

*Observations on the kidney*

In the original experiments, sections of kidneys of one of two rats killed at 9 weeks, and of all rats killed more than 9 weeks after the start of the injections show a remarkable picture of apparent necrosis of the proximal convoluted tubules. The affected tubules show complete disintegration of both cytoplasm and nucleus leaving only amorphous, lightly staining material within an apparently intact

basement membrane (Fig. 1). The acute nature of this renal lesion and the complete absence of tissue reaction are not in keeping with the duration of the experiment, and good health and normal renal function of the animals. We concluded that the tubular changes had developed after death. This has been confirmed in the present experiments where the right kidney, fixed immediately, invariably shows a normal histological appearance whereas the left kidney, fixed after an interval, shows extensive tubular autolysis. A study of the time relationships involved has shown that autolysis begins in isolated proximal tubules at all depths in the cortex 1 to 1½ hr. after death (Fig. 2); the changes are more extensive at 2 hr. and by 3 hr. most of the proximal and many of the distal tubules have disintegrated (Fig. 1). Little further change is seen 6 and 24 hr. later.

Kidneys from normal animals or control rats injected with dextran do not display this pattern of autolysis even after 24 hr. at room temperature. The only change occurring in these animals during the first 3 hr., is a surface effect involving shrinkage, early cytolysis and loss of eosinophilia in the outer 2 or 3 rows of proximal tubules (Fig. 3). This could be eliminated by anaerobiosis, by immersion of the slice of kidney in tris-(hydroxymethyl)-aminomethane buffer pH 7·2 under anaerobic conditions or in the presence of a reducing agent. None of these modifications had the slightest influence on the course of renal autolysis in the iron-loaded rat kidneys.

In vitamin E deficiency a similar accelerated autolysis is seen; Emmel (1955, 1957) has detected a rise in renal autolytic NPN level as early as the sixth week and he has reported that it becomes more striking with time. In the iron-loaded rats no significant difference from autolysing normal control kidneys was found when the autolysis was carried out in air at 18° or 37° for 6 hr. Houchin (1946) reported that the kidneys of hamsters deficient in vitamin E had a distinctly raised $Q_{O_2}$. This did not prove to be the case with iron-loaded rat kidneys.

*Uterus and testis*

The hypertrophied uterus, brown as a result of accumulation of ceroid pigment is a well-recognised feature of vitamin E deficiency (Martin and Moore, 1936). We first encountered it in fully developed form in rats killed 50 weeks after the start of iron-dextran injections. At 32 weeks the effect was macroscopically only a slight one, but ceroid was histologically demonstrable in myometrial fibres and macrophages.

Testicular atrophy was observed only in two instances, in rats killed at 47 and 52 weeks from the start of injections. The testes of 18 other iron-loaded male rats, killed at the same time or earlier in the experiment, were normal, as were those of 27 male rats of other groups given smaller total doses of iron and 14 dextran-treated controls. In the two affected animals the testes were obviously shrunken and show marked tubular atrophy, suppression of spermatogenesis and apparent hyperplasia of interstitial cells (Fig. 4). The interstitial cells are heavily loaded with haemosiderin but ceroid-like polymers are not conclusively demonstrated.

*Formation of polymeric pigments*

At least three different polymeric pigments have formed in our animals in various viscera. The more important histochemical features of the pigments are summarised in Table II.

TABLE II.—*Histochemical Characteristics of Polymeric Pigments*

| | H. & E. | Fe | Mallory haemo-fuscin | Ziehl-Neelsen | Periodic acid-Schiff | Acetyl Sudan Black B | U.V. fluores-cence | Neutral red tartra-zine |
|---|---|---|---|---|---|---|---|---|
| Pigment 1 (true ceroid) | Light grey | 0 | + | + | + | + | + | 0 |
| Pigment 2 (in kidney) | Light brown | 0 | ± | + | ± | 0 | ± | + |
| Pigment 3 (iron-containing polymer) | Orange | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Haemosiderin | Brown | + | 0 | + | + | 0 | 0 | 0 |

Type 1 polymer, classical ceroid, is seen in the cytoplasm of myometrial cells and in macrophages among these cells (Fig. 5), imparting to the uterus a dark brown colour which is a most striking feature at autopsy. A similar polymeric material is seen in smooth muscle of oviduct, vagina and small intestine, and in siderophages in most other viscera.

Type 2 polymer occurs in the epithelial cells of the proximal tubules of the kidney. This pigment is seen in very small amounts in some of our normal animals as fine granules within the tubular epithelial cells. It is present in far greater amounts in siderotic animals, appearing as large globules, frequently up to 15$\mu$ across, and lying apparently within the lumen of the tubule (Fig. 6). These globules are probably attached to the free border of the epithelial cells as they do not appear in the lumen of more distal tubules nor have we identified them in urinary deposits. The cortical tubular cells which contain this pigment usually contain small or moderate amounts of iron, but there is no correlation between the presence of polymer and development of autolysis : many necrotic tubules do not contain the polymer. Apart from small quantities of haemosiderin in tubular epithelium and stromal macrophages no pigment is found elsewhere in the kidneys.

Type 3 polymer is present chiefly in the liver. In iron-loaded animals, large siderophages appear in the hepatic sinusoids and in the portal tracts. Nearly all the animals killed 19 weeks or more following the start of injections show in many of these siderophages oval or irregular hyaline bodies, which vary greatly in size and stain bright orange with eosin (Fig. 7). This polymer is very occasionally present in siderophages in other viscera. Unlike the polymers listed above, this stains very heavily with the Prussian blue stain and is referred to in subsequent descriptions as " iron-containing polymer " (I.C.P.). It differs from haemosiderin in its failure to stain by the periodic acid-Schiff and Ziehl-Neelsen's techniques.

*Comparison with the vitamin E deficient rat*

Rapid post mortem renal autolysis, brown uterus, testicular atrophy and ceroid pigment formation are elements of the syndrome of vitamin E deficiency in the rat. A direct comparison was made with tissues of a rat maintained by Dr. T. Moore on a vitamin E deficient diet for 651 days. The following conclusions emerge :

1. There is a striking similarity in the picture of renal autolysis, which is rather more extensive and uniform in the vitamin deficient kidney.

2. There is much less ceroid in the kidney of the vitamin deficient rat. The globules are smaller, more disperse and irregular in outline.

3. The brown uterus of vitamin E deprivation shows a higher proportion of pigment in macrophages.

The suggestion of a relationship between the consequences of iron loading and vitamin E deficiency in the rat prompted a detailed search for confirmatory phenomena. The other features whose presence might have been anticipated in rats developing vitamin E deficiency on a low fat diet are: degeneration of muscles and nervous tissue, incisor depigmentation and susceptibility to haemolysis by dialuric acid (Dam, 1957).

Even before the vitamin E-deficient rat develops paralysis and neuromuscular disturbances, severe biochemical lesions are present in the muscles and histologically occasional damage to the fibres is seen (Mackenzie, 1949). In the iron-loaded rats a most careful scrutiny of the muscles likely to be affected failed to show any kind of degeneration. Biochemically, excessive creatinuria, phosphaturia and amino-aciduria were sought for, but all the results lay within the normal range. It should be recalled that the rats used in the experiments described here were well grown, whereas vitamin E depletion is usually undertaken in very young animals.

Methylene blue, when included in vitamin E-deficient diets, has proved capable of preventing for several months the development of brown discoloration of the uterus and renal autolysis (Moore, Sharman and Ward, 1953). Rats which had begun the course of Imferon injections 55 weeks before were given methylene blue (0·1 per cent) in the diet for 4 weeks. At the end of that time a definite retardation of renal autolysis was discernible. The uteri were only very pale brown in contrast to the dark colour in iron-loaded rats not given methylene blue. The thickening and dilatation which usually accompanies the brown colour were absent. Histologically there was diminution of ceroid in the uterus and some reduction also in the amounts of polymeric pigment present in liver and kidney.

### *Further Experiments*

Up to this point the experiments were carried out with rats which had been given iron up to a year or more before. It was desirable to study the relationship to vitamin E deficiency from the start of iron loading, and to accentuate the need for vitamin E by incorporating unsaturated fat in the diet. For this reason, in the present experiments, a moderate concentration of cod liver oil (10 per cent w/w) was incorporated in the diet. Polymer formation in various tissues of the original experiment was carefully reviewed.

The growth curves and liver weights of the experimental animals were very similar to those previously reported (Golberg *et al.*, 1957) for iron-loaded and control rats with initial weights in the range 100–120 g. The supplements given in the course of these experiments did not materially influence the basic effect of iron loading.

#### *Haemolysis tests*

Christensen *et al.* (1955) demonstrated that spontaneous haemolysis in isotonic saline at 37° was a more consistent and reliable test of vitamin E deficiency than the dialuric acid haemolysis test of Rose and György (1950). Our own findings (Table III) support these observations. By using the saline technique,

the effects of siderosis and of cod liver oil in promoting haemolysis are clearly shown, as is the protective part played by vitamin E.

TABLE III.—*Haemolysis of Red Cells from Individual Rats*

| Group | Degree of haemolysis* | |
|---|---|---|
| | Dialuric acid | Saline |
| Normal | 0, 0, 0, 0, 0, 0 | 0, 0, 0, 0, 0, 0 |
| $Fe_{16}$ | 0, 0, 1, 0, 0, 0 | 1, 1, 2, 0, 1, 0 |
| $FeE_{32}$ | 0, 0, 0, 2 | 1, 1, 0, 0 |
| $FeE_{32}O_{17-32}$ | 0, 0, 0, 0, 0 | 0, 1, 0, 0, 0 |
| $O_{32}$ | 0, 0, 0, 1, 0 | 1, 1, 1, 2 |
| $O_{32}E_{17-32}$ | 0, 0, 0, 0, 0 | 0, 0, 0, 0, 0 |
| $FeO_{32}$ | 0, 0, 0, 2, 0 | 2, 0, 1, 3, 2 |
| $FeO_{32}E_{17-32}$ | 1, 0, 0, 0 | 1, 0, 0, 0 |

* 0—absent; 1—faint but distinct; 2—moderate; 3—strong.

*Kidney*

The synergistic effect of iron loading and cod liver oil on the development of the propensity to renal autolysis could hardly have been demonstrated more clearly than in these experiments (Table IV). With cod liver oil alone renal autolysis was not observed even after 32 weeks. Iron loading alone produces only a slight degree of post mortem autolysis before 16–19 weeks. The two combined induced consistent and striking autolytic change at 8 weeks. Vitamin E, given from the start of the experiment, completely prevented autolysis resulting from iron-loading alone. In 4 out of 5 animals it prevented autolysis from developing in iron-loaded rats after 16 weeks of cod liver oil diet ($FeE_{32}O_{17-32}$). On the other hand when vitamin E was given in the latter half of the experiment ($FeO_{32}E_{17-32}$), it was able to restore to normal only one out of four of the rats' kidneys.

TABLE IV.—*Renal Autolysis and the Formation of Type 2 Polymer*

| Groups | Autolysis | Type 2 polymer* |
|---|---|---|
| Normal | 0, 0, 0, 0, 0, 0 | 0, +, ±, ±, ±, 0 |
| $Fe_8$; $Fe_{19}$ | 0, ±; +, +, +, + | 0, ±; +, ±, ++, ++ |
| $Fe_{32}$ | +, +, +, +, +, +, + | ++, ±, +, ++, ++, ++, ++ |
| $E_8$; $E_{16}$ | 0; 0 | 0; + |
| $FeE_4$; $FeE_8$; $FeE_{16}$ | 0, 0; 0, 0; 0, 0 | ±, 0; +, 0; ++, + |
| $FeE_{32}$ | 0, 0, 0, 0 | ±, +, ++, +++ |
| $FeE_{32}O_{17-32}$ | 0, 0, +, 0, 0 | ±, ++, +, ++, ++ |
| $O_4$; $O_8$; $O_{16}$; $O_{32}$ | 0; 0; 0; 0, 0, 0, 0, 0 | 0; ±; ±; ++, ±, +, +, ± |
| $O_{32}E_{17-32}$ | 0, 0, 0, 0, 0, 0 | ±, +, +, ±, ±, ± |
| $FeO_4$; $FeO_8$; $FeO_{16}$ | 0, 0; +, +; +, + | ±, ±; ±, ±; +, ++ |
| $FeO_{32}$ | +, +, +, +, + | +++, +++, +++, +++, +++ |
| $FeO_{32}E_{17-32}$ | 0, +, +, + | +++, +++, +++, +++ |

* The symbols indicate increasing amounts from traces (±) to heavy deposits (+++).

Despite the failure of our earlier efforts to demonstrate an abnormal increase in NPN during autolysis we tried again in the new series, with longer periods of incubation *in vitro*. Now a rise in NPN beyond the normal range could be shown to occur, but several kidneys which did not show autolysis histologically gave high values and conversely in a few instances striking autolysis was not

accompanied by very large NPN increments. Although it would be anticipated that the widespread autolysis would be associated with some rise in NPN, it may well be that the greater proportion of the autolytic products are peptides precipitable by trichloroacetic acid, which thus escape notice. In other words a strict parallel between autolysis and rise of NPN need not necessarily be expected.

The question of renal tubular type 2 polymer is most complex. Small amounts of this pigment are present as small granules in the proximal convoluted tubules of many normal rats in our colony. They were found even in one rat which had had vitamin E supplement to its normal diet for 16 weeks. All that can be said is that the amount of pigment present undergoes a great increase from 16 weeks of iron loading onwards, especially if there is added cod liver oil in the diet. There is a suggestion that tocopherol slightly retards its formation ; the vitamin certainly has no apparent effect on type 2 pigment once it has formed.

*Liver*

The effects of vitamin E and cod liver oil upon polymer formation and iron deposition in the liver are summarised in Table V. There is no significant influence on the amounts of I.C.P. in the siderophages ; the presence of this material appears to be associated only with prolonged accumulation of iron in the cell. Type 1 polymer occurs for the most part in small amounts in liver siderophages and parenchyma of iron-treated animals at 32 weeks. When vitamin E is given simultaneously with the iron no type 1 polymer is seen. With cod liver oil and iron, far greater amounts of polymer develop much earlier in the experiments. Even in the iron-vitamin E-treated group, supplementation of the diet with cod liver oil during the latter half of the period of study overcomes the protective action of the vitamin.

TABLE V.—*Summary of Polymer Formation and Iron Deposition in the Liver*

| Groups | I.C.P. in siderophages | Type 1 polymer | | Iron deposition | |
|---|---|---|---|---|---|
| | | Siderophages | Parenchyma | Siderophages | Parenchyma |
| $Fe_0$; $Fe_{16}$; $Fe_{32}$ | 0; +; ++ | 0; 0; ++ | 0; 0; ± | ++; ++; ++ | ±; +; ++ |
| $E_0$; $E_{16}$ | — | — | 0; 0 | — | — |
| $FeE_4$; $FeE_8$; $FeE_{16}$ | 0; 0; ± | 0; 0; 0 | 0; 0; 0 | +; +; ++ | +; ++; ++++ |
| $FeE_{32}$ | ++ | 0 | 0 | ++ | ++++ |
| $FeE_{32}O_{17-32}$ | ++ | + | + | ++* | +++ |
| $O_4$; $O_8$; $O_{16}$ | — | — | 0; 0; 0 | — | — |
| $O_{32}$ | — | — | 0 | — | — |
| $O_{32}E_{17-32}$ | — | — | 0 | — | — |
| $FeO_4$; $FeO_8$; $FeO_{16}$ | 0; 0; + | 0; +++; +++ | 0; +; + | +; ++; +++ | ±; ±; + |
| $FeO_{32}$ | +++(+) | +++ | + | +++* | ++ |
| $FeO_{32}E_{17-32}$ | ++ | +++ | + | ++* | ++++ |

* Tendency for siderophages to aggregate around central veins and portal tracts.

The distribution of iron between liver siderophages and parenchymal cells is not altered by the presence of cod liver oil in the diet. By contrast, vitamin E brings about a remarkable increase in the parenchymal iron. The only noticeable effect of cod liver oil is to enhance the time taken for the siderophages to be cleared from the sinusoids in order to form aggregations around the portal tracts and central viens. We had not previously noted migration of this kind in iron-loaded animals until about the fortieth week (Golberg *et al.*, 1957).

*Vitamin A storage in the liver*

Estimations were carried out on untreated control rats and on rats killed after 32 weeks of treatment. The normal rats have a relatively poor reserve of vitamin A in the liver (Table VI). At such low levels the differences between groups are rendered insignificant by the error in the estimation. In animals given cod liver oil the stores of vitamin A are naturally considerably increased. The effect of simultaneous iron-loading is distinctly to reduce these stores, despite the larger size of the liver (Golberg *et al.*, 1957). The protective action of vitamin E is clearly demonstrated and again iron loading reduces the vitamin A level. Equally clear-cut differences are seen if the results are expressed as total vitamin A in the whole liver or as total vitamin A per 100 g. body weight.

TABLE VI.—*The Vitamin A Content of Rat Livers*

| Group | Number of rats | Vitamin A, in i.u. per g. of liver* | | |
|---|---|---|---|---|
| | | Range | Mean | S.D. |
| Normal | 10 | 21–55 | 31 | 11 |
| $Fe_{32}$ | 4 | 18–38 | 28 | 9 |
| $FeE_{32}$ | 4 | 13–75 | 45 | 26 |
| $O_{32}$ | 5 | 700–1750 | 1340 | 418 |
| $FeO_{32}$ | 5 | 190–900 | 463 | 275 |
| $O_{32}E_{17-32}$ | 6 | 2050–4250 | 3025 | 797 |
| $FeO_{32}E_{17-32}$ | 4 | 100–350 | 207 | 106 |

* Measured at 32 weeks after the start of the experiment.

*Uterus and testis*

In iron-loaded rats on diet 41 alone the uteri appeared normal at 19 weeks and even at 32 weeks were only faintly brown. With cod liver oil as the sole supplement the uteri appeared either normal or pale yellow-brown at 32 weeks; only in one instance was there definite thickening and dilatation. Iron-loading in conjunction with dietary cod liver oil induced, from 8 weeks onwards, distinctly brown uteri. Besides the thickened dilated walls there was hydrometra in many cases and type 1 polymer was present in the uterine wall, both in myometrium and macrophages.

EXPLANATION OF PLATE.

FIG. 1.—Complete autolysis of proximal tubules of the kidney of an iron-laden rat after keeping in air at room temperature for 3 hours prior to fixation. P.A.S. × 340.

FIG. 2.—Less advanced autolysis of renal tubules after keeping for 2 hours. P.A.S. × 450.

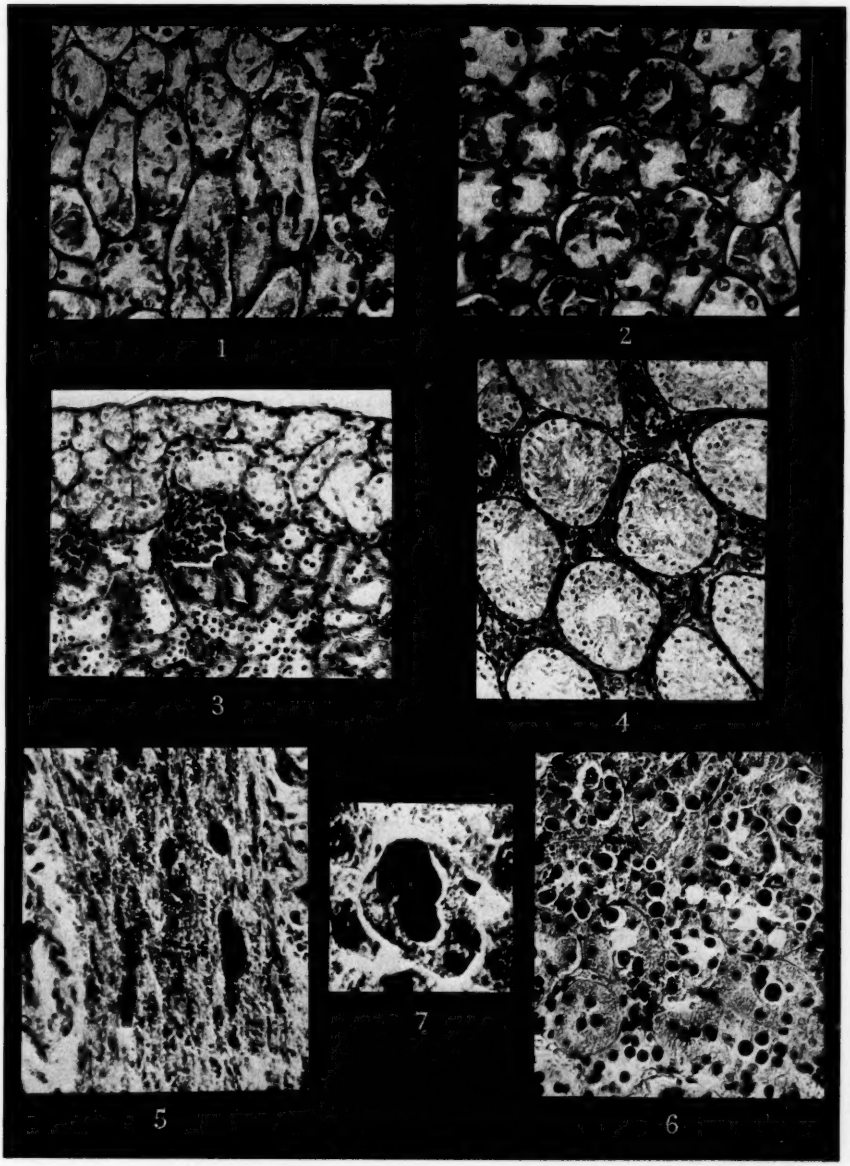
FIG. 3.—Surface shrinkage and minor cytolysis in outer rows of tubules only of the kidney from a normal rat kept in air for 3 hours prior to fixation. H. & E. × 190.

FIG. 4.—Testis from a rat 52 weeks following a course of iron injections. The tubules are shrunken and spermatogenesis is absent; there is hyperplasia of interstitial cells. H. & E. × 110.

FIG. 5.—Fine granules of ceroid polymer in myometrial cells and interstitial macrophages of an iron-laden rat. P.A.S. × 500.

FIG. 6.—Rounded globules of ceroid polymer in renal tubular epithelium of an iron-laden rat. Neutral red-Tartrazine. × 400.

FIG. 7.—An irregular mass of iron-containing polymer (I.C.P.) in a siderophage in the liver of an iron-laden rat. H. & E. × 810.




Golberg and Smith.

Demole (1941) has claimed that prolonged administration of tocopheryl acetate will completely restore the brown uterus to normal. In general, however, vitamin E is believed to have a prophylactic rather than a curative action. In these experiments vitamin E neither prevented nor completely cured the degenerative uterine changes, though it did serve to reduce the amount of ceroid present (Table VII).

TABLE VII.—*Summary of the Occurrence of Type* 1 *Polymer in Various Organs of the Rat*

| Group | Uterus | Body fat | Small intestine: Villi | Small intestine: Circular muscle | Lymph nodes |
|---|---|---|---|---|---|
| $Fe_{32}$ | + | 0 | ± | 0 | ± |
| $FeE_{32}$ | 0 | 0 | 0 | 0 | 0 |
| $FeE_{32}O_{17-32}$ | 0 | ± | ++ | ± | ± |
| $O_{32}$ | ± | + | ++ | 0 | ++ |
| $O_{32}E_{17-32}$ | 0 | 0 | + | 0 | ± |
| $FeO_{32}$ | ++ | ++ | +++ | + | ++ |
| $FeO_{32}E_{17-32}$ | + | ++ | ++ | + | ++ |

Further evidence of the synergistic effect of cod liver oil and tissue siderosis was forthcoming from observations on the testis. Atrophy of the seminiferous tubules developed *only* in the rats of group $FeO_{32}$. The complete absence of atrophy in the testes of rats of other iron-loaded groups is particularly striking in view of the equal deposition of iron in interstitial cells. The presence of vitamin E in adequate amounts in diet 41 may have contributed to this clear-cut result.

*Body fat*

The body fat of our original iron-loaded rats which consumed a diet of low lipid content showed no abnormality. On a diet incorporating 10 per cent cod liver oil there developed, after 32 weeks, distinctly brown fat in 2 out of 5 rats. Histologically, type 1 polymer was present in the subcutaneous fat of only one, but in the lumbar fat of all 5. These changes were absent in animals which had had vitamin E over the last 16 weeks ($O_{32}E_{17-32}$).

Even though there is practically no histologically demonstrable iron in the intra-abdominal fat, iron-loading greatly intensified both the brown colour and the amount of type 1 ceroid present in both lumbar and subcutaneous fat (Table VII). In $FeO_{32}$ rats, dosing with vitamin E during the last 16 weeks produced fat which did not appear distinctly brown ; but histologically ceroid was present in all specimens of subcutaneous and lumbar fat. Group $FeE_{32}$ had normal fat ; of the 5 rats in group $FeE_{32}O_{17-32}$, one showed pale brown fat, containing type 1 ceroid.

It is clear that when unsaturated fat is present in the diet iron-loading accentuates the antagonism to vitamin E ; and that treatment with additional vitamin E eliminates the effects of the unsaturated lipid and of the iron. These phenomena were demonstrated by chemical and histochemical tests for lipoperoxides. Chemical analyses of peroxides in subcutaneous, lumbar and periovarial or paraepididymal fat showed that occasionally there was a considerable variation from one sample to another of fat from the same region of the same animal. Thus in one rat from group $FeO_{32}$ five specimens of subcutaneous fat gave the following values (in

m. equiv. peroxide/kg. fat): 1·8, 2·1, 2·8, 9·7, 42·2. Much of the fat was greatly altered, as described by Mason, Dam and Granados (1946). For lumbar fat of the same animal the values were 5·7 and 6·2, for perigonadal fat 4·0. The normal range by our method was 0·3–2·2. Each of the animals in the groups which had had cod liver oil showed at least one value beyond the normal range. Vitamin E had only a slight effect in reducing the peroxide content of the fat. In the case of the $FeO_{32}$ group the peroxide figures for subcutaneous, lumbar and perigonadal fat were respectively (range and mean of whole group): 1·8–42·2 (6·0), 1·4–17·7 (6·3) and 1·6–7·8 (3·8); whereas for the $FeO_{32}E_{17-32}$ group the figures were: 1·5–2·2 (1·9), 1·4–6·8 (2·9), 1·1–3·0 (1·9).

Histologically a positive peroxide reaction could be elicited in formalin-fixed material. The intensity of the pink colour produced was greatest in the $FeO_{32}$ group but no regular correlation was found between the histochemical test and chemical estimations. This is hardly surprising in view of the transient existence of peroxides in tissues and is in accordance with the findings of Dam and Granados (1945). It should be noted that haemosiderin granules do not give a positive reaction, nor are they associated with the presence of peroxides in their immediate neighbourhood.

*Type* 1 *polymer in other organs*

Table VII shows the effects of vitamin E and cod liver oil on the development of type 1 polymer in the villi of the small intestine and lymph nodes. Ceroid polymer is seen in macrophages and in smooth muscle cells in the cores of the villi of the small intestine where small accumulations of haemosiderin also occur. Less commonly the polymer is seen in the cells of the circular muscle of the intestinal wall. Lymph nodes throughout the body contain much iron in large siderophages: these cells, especially in intra-abdominal lymph nodes frequently show small and sometimes large accumulations of ceroid. As in the liver, vitamin E inhibits whilst cod liver oil enhances the formation of polymer.

## DISCUSSION

*Intracellular iron*

The belief has gained credence that haemosiderin is a relatively inert material, incapable of contributing to, or participating in, the body's iron pool. Heilmeyer (1957) and others have asserted that the iron of haemosiderin is not available to the body in the same way as that of ferritin. In fact it was demonstrated by Shoden, Gabrio and Finch (1953) that " these two types of storage iron are intimately associated and functionally indistinguishable ". Haemosiderin consists of large agglomerations of iron micelles and ferritin itself may be a component of haemosiderin granules (Richter, 1957*a* and *b*). The iron granules are set in a matrix composed of mucopolysaccharide, lipid and protein (Goessner, 1953; Gedigk and Strauss, 1953, 1954). Ferritin molecules themselves are composed of micelles of ferric hydroxide and phosphate (Granick, 1945). According to Laurell (1957) there exists a " diffusion pressure " of ionic iron in tissues, maintaining an equilibrium between different cells of the body. A dynamic relationship exists between the iron ions within the ferritin micelle, on the surface of the micelle, in solution in the intracellular fluid and bound to transferrin in the plasma.

Abnormally heavy accumulations of ferritin and haemosiderin within cells may bring about effects mediated by high localised concentrations of unbound ionic iron. An effect of this kind is the influence, direct or indirect, on vitamin E. Despite adequate dietary levels of this vitamin, the pathological and biochemical consequences of deficiency appear in those organs and at those sites where iron accumulates preferentially, while other organs and tissues which would be affected by an overall deficiency of vitamin E remain unscathed.

*Localisation of lesions resulting from iron-loading*

The high uptake of $Fe^{59}$ in the kidney, more particularly in the proximal convoluted tubules, was first noted by Finch *et al.* (1950). Bergström, Magnusson, Odeblad and Ziliotto (1955) demonstrated with injected $Fe^{59}$, not only that the proximal convoluted tubules always took up a great deal of iron, but that the amount increased until the conclusion of the experiment 9 days later. Our own observations that iron accumulates in the same site during iron-loading would suggest the presence in the tubular epithelium of some iron-binding material from which, perhaps under favourable circumstances, the large amounts of ceroid can develop.

Organs which are known to accumulate iron both as a result of reticulo-endothelial activity or as part of the normal ageing process were similarly affected by iron-loading ; again ceroid formation was a prominent feature. Although the muscular changes of vitamin E deficiency do not develop in the adult rat until an advanced age, some evidence might have been expected in our rats if the effect of iron-loading were a generalised one, *i.e.* induction of an overall vitamin E deficiency. The fact that a careful search for muscle lesions did not reveal any does suggest that lack of iron deposition in muscle was responsible. Even the earliest sign of an effect on muscle, namely creatinuria, was not present.

*Iron and vitamin E*

Evidence for a connection between iron and vitamin E has existed for many years. The oxidation of tocopherols with ferric chloride, giving rise to ferrous ions, is the basis of the Emmerie-Engel method of estimating vitamin E. Under carefully controlled conditions $\alpha$-tocopherol is converted by ferric chloride *in vitro* to the unstable intermediate $\alpha$-tocopheroxide, further oxidation of which leads to inactivation (Boyer, 1951 ; Harrison, Gander, Blakley and Boyer, 1956).

The biological consequences of the interaction of iron with vitamin E were demonstrated by Waddell and Steenbock (1928, 1931) who added ferric chloride in solution in ether to a stock ration and subsequently aerated it. This diet rendered male and female rats infertile, while no such effect was manifested when the iron-treated diet was supplemented by daily doses of wheat-germ oil or when the iron was added to the diet in aqueous solution without aeration. Goettsch and Pappenheimer (1931) used a similar method for inducing nutritional muscular dystrophy in the guinea-pig and rabbit.

King, Lee and Visscher (1955*a*) found that mouse paralysis attributable to lack of vitamin E developed on a diet containing ferrous sulphate, but did not do so when no iron was provided or when the iron was in the form of " ferric phosphate soluble ". The importance of ionised as opposed to non-ionised iron was demonstrated in a further paper (1955*b*) in which cardiac lesions due to vitamin E deficiency

were seen in mice given a diet whose salt mixture contained ferrous sulphate, while no such lesions were obtained with " ferric phosphate soluble " or on a normal diet. The influence which ionic iron and copper have on oxidative processes is well-known and, as Cummings and Mattill (1931) have said, " the efficiency of a given source of vitamin E depends in part on the auto-oxidizable materials and anti-oxidants associated with it ".

Despite its extensive use and satisfactory performance under standard laboratory conditions, diet 41 may have shortcomings as revealed by Bruce (1950). He found that an increase in the content of cod liver oil from 1 to 2 per cent served to induce vitamin E deficiency in mice fed on this diet. The tocopherol content of diet 41 used in the present experiments is (in mg./100 g. food) $\alpha$ 0·52 ; $\beta$ 0·16 ; $\zeta$ 0·31 ; $\epsilon$ 0·35. The total falls short of the value of 3 mg./100 g. food assessed by Brown and Sturtevant (1949) to be the vitamin E requirement of the growing rat. Our rats were somewhat older than those usually employed in vitamin E studies ; also some of the published values place the tocopherol requirement much lower—for instance 0·75 mg. $\alpha$-tocopherol/100 g. food protects the male and female rat from sterility (Mason, 1940). It follows that diet 41 may be regarded as adequate with respect to vitamin E under ordinary circumstances, but not perhaps when tissue siderosis is present.

*Iron and vitamin A*

Why vitamin E is more sensitive than vitamin A to the influence of ionic iron is uncertain but it is a fact established by Waddell and Steenbock (1928) and others. The interesting story of " salt ophthalmia " or vitamin A deficiency resulting from the presence of ferrous sulphate in the diet (Jones, 1927) cannot be recounted here. Suffice it to say that the ophthalmia could be prevented or cured, by replacing ferrous sulphate by the relatively unionised salt ferric citrate, by giving wheat-germ oil, or by making up fresh ferrous sulphate diet each day, thus avoiding prolonged contact of oxidisable fats with ferric ions (McCollum, Simmonds and Becker, 1927).

Vitamin E has a protective effect on vitamin A in the intestinal tract (Hickman, Harris and Woodside, 1942) and in the liver (Davies and Moore, 1941). Thus our finding of decreased liver stores of vitamin A in siderotic animals could be interpreted either in terms of a direct action of intracellular iron on vitamin A or as an indirect effect of destruction or antagonism of vitamin E. This question cannot be resolved with the data available but the fact that the $FeO_{32}E_{17-32}$ groups had no higher levels of liver vitamin A than the $FeO_{32}$ group does suggest a direct and continuing action of iron on the vitamin A in the liver. It may be that vitamin E administered orally cannot accumulate in the liver in sufficient amounts to combat the very high localised intracellular concentrations of iron. Reticuloendothelial blockade lowers the vitamin A content of the liver (Krishnamurthy and Ganguly, 1956), but there is little likelihood that such blockade would be manifested six months after the injections of iron-dextran had ceased.

*Evidence contributed by the present experiments*

The effects which have been described here could be exercised by the large accumulations of intracellular iron through one or more of the following

mechanisms: oxidative destruction of vitamin E (and, possibly, vitamin A); direct or indirect (catalytic) participation in the formation of peroxidised lipids; direct oxidation of sulphydryl groups; a generalised disturbance of oxidation-reduction potential in the affected cells.

Like many others, Haurowitz, Schwerin and Yenson (1941) demonstrated the action of haemoglobin and haemin on autoxidation of unsaturated fatty acids. Attempts to repeat these observations with ferritin have shown only a slight catalytic effect (Golberg and Pickering, 1957). However the experiments described here point to the pro-oxidant activity of iron by demonstrating the presence of lipid peroxides and decreased liver stores of vitamin A. Even if the iron-laden tissues were found to contain less tocopherol than normal, it would scarcely resolve the problem. Hove (1955) has pointed out that "reliance on pathologic similarity as an indication of vitamin E deficiency or antagonism may be misleading". He defines a "stress factor" as "any substance that precipitates a pathological condition curable or preventable by vitamin E". Iron under these circumstances could presumably be acting as a "stress factor", having this property in common with tri-*o*-cresyl phosphate, pyridine and a host of other compounds. This is unlikely to be the complete explanation since it fails to take account of ceroid formation—the large accumulations of various polymers at sites of iron deposition. In the case of the kidney these accumulations greatly exceeded any hitherto described.

The mode of formation of ceroid and its significance are as yet little understood. Pigments corresponding to haemosiderin and our types 1 and 2 polymers were described by Edwards and White (1941) in rats fed butter yellow. These authors suggested that the renal ceroid was the same as the pigment reported by Fairhall and Miller (1941) in the kidneys of rats given lead compounds. Hepatic (type 1) ceroid was also reported in rats subjected to chronic copper poisoning (Mallory, Parker and Nye, 1921) and in mice in whom necrosis was induced by ligation of the left lower lobe of the liver (Lee, 1951). The latter author showed striking chronological alterations in the histochemical reactions of the ceroid. Initially it was iron-positive but by the time the characteristic staining properties of the polymer were fully developed the iron reaction had become variable.

Is there a common basis underlying all these methods of ceroid production? It would appear that the union of iron and lipid will, under appropriate circumstances, lead to ceroid formation. The iron may be present as a result of haemorrhage, necrosis (with resulting liberation of iron from intracellular enzymes) or accumulation of ferritin and haemosiderin beyond physiologically normal limits. Perhaps the heavy metals copper and lead can assume the role of iron in stimulating the production of ceroid by acting as pro-oxidants; or they may liberate free iron indirectly by damaging the mitochondria or other cellular components. Unsaturated lipid could arise from pathological accumulations, as suggested by Casselman (1951) or, more simply, from damaged cellular components. The circumstances appropriate for ceroid formation have been defined by Casselman as a "relative, if not concurrent absolute, deficiency of biological antioxidants". A preponderance of heavy metal, particularly iron or copper, would fill the bill.

The hypothesis that tissue siderosis exercises pathological effects in parenchymal and other cells by changing the intracellular oxidation-reduction potential opens up many avenues for further investigation.

SUMMARY

A gross degree of iron overload has been induced in rats on M.R.C. diet 41 by the administration of a total of 1·65 g. Fe/kg. body weight, given intramuscularly in doses of 75 mg. Fe/kg. three times a week. The sequelae closely resemble many of the characteristic pathological and biochemical changes observed in rats deficient in vitamin E.

Many of these changes are accentuated by the incorporation of cod liver oil in the diet, while vitamin E exercises a protective action. The absence of evidence of total body deficiency of vitamin E is attributed to the preferential accumulation of iron in certain sites.

Three types of polymeric pigment are described and their mode of formation briefly discussed.

Our thanks are due to the staffs of our respective departments for technical assistance, to Dr. T. Moore for providing tissues from a vitamin E deficient rat and to Dr. J. Green for carrying out the analysis of tocopherols in diet 41. We are indebted to Professor A. C. P. Campbell for his interest and advice and to the Directors of Benger Laboratories Ltd. for permission to publish the results of our investigations.

## REFERENCES

Bergström, I., Magnusson, G., Odeblad, E. and Ziliotto, D.—(1955) *Acta physiol. scand.*, **35**, 36.
Boyer, P. D.—(1951) *J. Amer. chem. Soc.*, **73**, 733.
Brown, R. A. and Sturtevant, M.—(1949) *Vitam. & Horm.*, **7**, 171.
Bruce, H. M.—(1950) *J. Hyg., Camb.*, **48**, 171.
*Idem* and Parkes, A. S.—(1949) *Ibid.*, **47**, 209.
Casselman, W. B.—(1951) *J. exp. Med.*, **94**, 549.
Christensen, F., Dam, H., Görtner, R. A. Jr. and Sondergaard, E.—(1955) *Acta physiol. scand.*, **35**, 215.
Cummings, M. J. and Mattill, H. A.—(1931) *J. Nutr.*, **3**, 421.
Dam, H.—(1957) *Pharmacol. Rev.*, **9**, 1.
*Idem* and Granados, H.—(1945) *Acta physiol. scand.*, **10**, 162.
Davies, A. W. and Moore, T.—(1941) *Nature, Lond.*, **147**, 794.
Demole, V.—(1941) *Schweiz. med. Wschr.*, **22**, 1251.
Edwards, J. E. and White, J.—(1941) *J. nat. Cancer Inst.*, **11**, 339.
Emmel, V. M.—(1955) '3rd Int. Congr. Vitamin E, Abstr.', p. 31.—(1957) *J. Nutr.*, **61**, 51.
Fairhall, L. T. and Miller, J. W.—(1941) *Publ. Hlth Rep., Wash.*, **56**, 1610.
Finch, C. A., Hepsted, M., Kinney, T. D., Thomas, E. D., Rath, C. E., Haskins, D., Finch, S. and Fluharty, R. G.—(1950) *Blood*, **5**, 983.
Gedigk, P. and Strauss, G.—(1953) *Virchows Arch.*, **324**, 240.—(1954) *Ibid.*, **326**, 172.
Glavind, J., Granados, H., Hartmann, S. and Dam, H.—(1949) *Experientia*, **5**, 84.
Goessner, W.—(1953) *Virchows Arch.*, **323**, 685.
Goettsch, M. and Pappenheimer, A. M.—(1931) *J. exp. Med.*, **54**, 145.
Golberg, L.—(1957) *Postgrad. Med.*, **22**, 382.
*Idem*, Smith, J. P. and Martin, L. E.—(1957) *Brit. J. exp. Path.*, **38**, 297.
Granick, S.—(1945) *Chem. Rev.*, **38**, 379.
Harrison, W. H., Gander, J. E., Blakley, E. R. and Boyer, P. D.—(1956) *Biochim. biophys. Acta*, **21**, 150.

HARTMANN, S. AND GLAVIND, J.—(1949) *Acta chem. scand.*, **3**, 954.
HAUROWITZ, F., SCHWERIN, P. AND YENSON, M. M.—(1941) *J. biol. Chem.*, **140**, 353.
HEILMEYER, L.—(1957) *Clin. Chim. Acta*, **2**, 159.
HICKMANN, K. C. D., HARRIS, P. L. AND WOODSIDE, M. R.—(1942) *Nature, Lond.*, **150**, 91.
HOUCHIN, O. B.—(1946) 'Biol. Antioxidants Trans. 1st Conference', 60. Josiah Macy Foundation, New York.
HOVE, E. L.—(1955) *Amer. J. clin. Nutr.*, **3**, 328.
JONES, J. H.—(1927) *J. biol. Chem.*, **75**, 139.
KING, J. T., LEE, Y. C. P. AND VISSCHER, M. B.—(1955*a*) *Proc. Soc. exp. Biol., N.Y.*, **88**, 406.—(1955*b*) *J. Nutr.*, **57**, 111.
KRISHNAMURTHY, S. AND GANGULY, J.—(1956) *Nature, Lond.*, **177**, 575.
LAURELL, C.-B.—(1957) *Mem. Univ. Calif.*, in press.
LEE, C. S.—(1951) *J. nat. Cancer Inst.*, **11**, 339.
MACKENZIE, C. G.—(1949) *Ann. N.Y. Acad. Sci.*, **52**, 202.
MALLORY, F. B., PARKER, F. Jr. AND NYE, R. N.—(1921) *J. med. Res.*, **42**, 461.
MARTIN, A. J. P. AND MOORE, T.—(1936) *Chem. & Ind.*, **55**, 236.—(1939) *J. Hyg., Camb.*, **39**, 643.
MASON, K. E.—(1940) *Amer. J. Physiol.*, **131**, 268.
*Idem*, DAM, H. AND GRANADOS, H.—(1946) *Anat. Rec.*, **94**, 265.
MCCOLLUM, E. V., SIMMONDS, N. AND BECKER, J. E.—(1927) *Proc. Soc. exp. Biol., N.Y.*, **24**, 952.
MOORE, T., SHARMAN, I. M. AND WARD, R. J.—(1953) *Biochem. J.*, **53**, xxxi.
RICHTER, G. W.—(1957*a*) *Amer. J. Path.*, **33**, 590.—(1957*b*) *J. exp. Med.*, **106**, 203.
ROSE, C. S., GYÖRGY, P.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **74**, 411.
SHODEN, A., GABRIO, B. W. AND FINCH, C. A.—(1953) *J. biol. Chem.*, **204**, 823.
WADDELL, J. AND STEENBOCK, H.—(1928) *Ibid.*, **80**, 431.—(1931) *J. Nutr.*, **4**, 79.

# COMPARATIVE SEROLOGICAL STUDIES ON TALFAN AND TESCHEN DISEASES AND SIMILAR CONDITIONS

DONNA M. CHAPRONIERE, J. T. DONE AND C. H. ANDREWES

*From the National Institute for Medical Research, Mill Hill, London, N.W.7 and the Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, Surrey*

Received for publication November 1, 1957

A POLIO-ENCEPHALOMYELITIS affecting young pigs in Britain has lately been described by Harding, Done and Kershaw (1957) under the name of Talfan disease. The disease clearly showed similarities to Teschen disease, an infection of pigs well known in central Europe and Madagascar, and to poliomyelitis suum (Bendixen and Sjolte, 1955). It seemed highly probable that the Talfan virus, like Teschen virus (Larski, 1955 ; Mayr and Schwobel, 1956) would prove to be propagable in tissue culture : if so a ready means of serological comparison of these viruses would be at hand. Our expectations were quickly realized and the results of cross-neutralization tests are here briefly reported.

## MATERIALS AND METHODS

### *The virus*

*Talfan.*—Virus in the form of infected pig brain was available from animals inoculated in the transmission experiments described by Harding *et al.* (1957). The virus was propagated in tissue culture from 1st and 2nd pig passage material.

*Teschen.*—Dr. E. Traub (Tübingen, Germany) kindly sent Teschen virus (Konratice strain) at the 2nd and 58th subculture level. The latter was used in these experiments.

### *Sera*

*Talfan.*—Sera from one hyperimmunized pig and several pigs convalescent after experimental infections were available.

*Poliomyelitis suum.*—Pig sera immune to poliomyelitis suum were kindly provided by Dr. Aa. Thordal-Christensen (Copenhagen).

*Teschen.*—Hyperimmune anti-Teschen pig serum was very kindly provided Dr. D. Horstmann (New Haven, U.S.A.) (1 Swedish and 3 Czechoslovak strains) ; other Teschen immune pig sera by Dr. E. Traub and Dr. F. Patoĉka (Prague) and poliovirus serum from immunized monkeys by Dr. J. O'H. Tobin.

*Enzootic ataxia* (Done 1957).—Serum was available from a naturally occurring case of the disease in an Essex pig. The serum was obtained 1½ years after the onset of clinical disease.

### *Tissue culture methods*

Roller tube tissue cultures of pig kidney were used for this investigation. Fragments of kidneys from pigs of various ages were either explanted into a plasma clot on to the wall of a test tube (5 explants per tube) or were trypsinized by the method of Dulbecco modified by Balducci, Zaiman and Tyrrell (1956) and allowed to grow until a sheet of cells formed. The medium used for experiments with virus was composed of 5 per cent inactivated horse serum ; 0·25 per cent lactalbumin hydrolysate ; 40 per cent bovine amniotic fluid ; 54·75 per cent Gey's solution ; 100 units/ml. sodium penicillin G ; 100 μg/ml. dihydrostreptomycin; (in cultures with plasma) 0·1 mg./ml. soya bean trypsin inhibitor and 0·01 mg./ml. phenol red.

*Neutralization tests*

The medium was removed from well-grown cultures of either kind and replaced by 0·8 ml. of fresh medium. Of the serum under test 0·1 ml. was then added to each of a group of 9 tubes and the cultures allowed to rotate in the drum for 10–20 min. Then dilutions of virus were added to groups of 3 tubes ; thus each serum was set up in triplicate against 3 dilutions of virus. The tubes were observed for 8 days. Previous experiments showed that if Talfan virus was present it would destroy the cultures within 1–8 days

## EXPERIMENTAL

Virus derived from a case of Talfan disease was passaged ten times in pig kidney cultures. Fifth pass material was used as stock for subsequent experiments. The titre of virus at each pass tested in baby pig kidney was between $10^6$ and $10^{7.5}$ tissue culture infectious doses ($T.C.I.D._{50}$) per ml. Adult pig kidney was less sensitive to virus. The virus destroyed the cells in 1–8 days, according to the dilution used as inoculum. Attempts to grow the virus in guinea-pig kidney, rat embryo, rabbit kidney cultures and in human liver cells (Chang line) and HeLa cells were unsuccessful, but the decay rate of virus in such cultures was very slow.

Talfan virus resisted heating at 56° for 3 hr. with a diminution of titre of less than 1000 times. The end-point of the titration was not reached, however, and the loss of virus may have been several logs less. Passes were made from cultures infected with heated virus, which showed that the destruction of the cells was due to live virus and not a toxic effect of killed virus. This virus also withstood 20 per cent ether treatment overnight at 4° with no significant loss of titre.

Talfan tissue culture virus at the 2nd and 5th subculture levels was inoculated intracerebrally into 3½-week-old litter-mate pigs as shown in Table I. The resultant disease differed from that produced experimentally with freshly isolated virus (Harding *et al.*, 1957) only in the higher morbidity rate and shorter incubation period ; clinically and histopathologically it was identical with Talfan disease.

Brain and cord emulsion from a pig killed at the onset of clinical disease (PD 3160) on the 6th day was inoculated into a further 4 piglets in which typical Talfan disease resulted. It was presumed that the very short incubation period

TABLE I.—*Experimental Infections in Pigs with Tissue Culture Talfan Virus*

| Pig No. | Inoculum | Days to clinical disease | Days to death | Killed/Died | Histological result |
|---|---|---|---|---|---|
| PD 3184 | 5th pass tissue culture Talfan virus | 10 | 14 | K* | + |
| PD 3186 | | 11 | 16 | K* | + |
| PD 3187 | | 15 | 16 | K* | + |
| PD 3199 | | 11 | 18 | D | + |
| PD 3152 | None | — | 0 | K | — |
| PD 3160 | 2nd pass culture virus | 6 | 6 | K | + |
| PD 3181 | | 7 | 10 | D | + |
| PD 3210 | None (contact control) | — | 21 | K | — |
| PD 3209 | Emulsion of C.N.S. of PD 3160 | 10 | 15 | K* | + |
| PD 3213 | | 10 | 15 | D | + |
| PD 3214 | | 10 | 13 | K* | + |
| PD 3288 | | 11 | 50 | K | + |
| PD 3233 | None (contact control) | — | 28 | K | — |

* Killed *in extremis*.

seen with 2nd pass tissue culture virus was a function of the virus concentration in the inoculum rather than due to a fundamental change in the virus.

### *Relationship to Teschen Disease*

The cytopathic effect of Talfan virus on pig kidney cultures is similar to that described by Mayr and Schwobel (1957) for Teschen virus. Talfan virus also resembles their virus in its high resistance to heat and to ether.

The disease produced by experimental infection of pigs with tissue culture Talfan virus more closely resembles classical Teschen disease in its high morbidity and mortality and shorter incubation period than Talfan disease as seen in Britain or poliomyelitis suum as described in Denmark. This parallels the observations by Mayr (1957) on Teschen virus.

Neutralization tests in tissue culture (Table II) showed that the cytopathic effect of Talfan virus was neutralized by 6 samples of Teschen antisera obtained from different countries, by poliomyelitis suum antiserum (Bendixen and Sjolte, 1955) from Denmark and by enzootic ataxia serum. It was not neutralized by human poliomyelitis antiserum of any of the 3 types. Talfan antiserum also neutralized Teschen virus in culture. Differences in potency between the various sera, obtained under varying conditions, are not thought likely to be of great significance.

TABLE II.—*Neutralization Tests with Talfan and Teschen Disease Viruses in Tissue Culture*

| Virus | Serum | No. of $LD_{50}$ neutralized (logs) |
|---|---|---|
| Talfan | Talfan | 1·7 |
| Teschen | " | 3 |
| Talfan | Teschen | |
| | (a) Konratice (Traub) | 3 |
| | (b) Konratice (Horstmann) | 1·2 |
| | (c) Reporyje | 1·2 |
| | (d) Vetisuci | >2 |
| | (e) Swedish | >2 |
| | (f) Patočka* | 2·3 |
| Talfan | Enzootic ataxia | >2 |
| Talfan | Polio suum | 2·2 |
| Talfan | Polio type I | <1 |
| | II | <1 |
| | III | <1 |

* Serum diluted 1/100. Other sera diluted 1/10.

## DISCUSSION

Neutralization tests in culture and experimental infections with tissue culture virus demonstrate that Talfan disease and Teschen disease are caused by identical or closely similar viruses, which also are closely related to or identical with " poliomyelitis suum " from Denmark.

We feel it important that attention should be drawn to this similarity. The disease described by Harding *et al.* (1957) under the name Talfan disease is milder

than the disease described under the title of Teschen, but its virulence seems to be similarly enhanced by tissue culture. No epidemics have so far occurred in Britain, but according to Patoĉka (personal communication) only sporadic cases were at first observed in Czechoslovakia, the disease now being endemic with occasional epidemic outbursts.

SUMMARY

A virus causing paralysis in pigs in Britain (Talfan disease), was propagated in cultures of pig kidney : cultures reproduced the disease in pigs when inoculated intracerebrally.

Talfan virus was neutralized by antisera active against strains of Teschen disease from Czechoslovakia, Sweden and Germany ; also by sera from a case of enzootic ataxia in Britain and of " poliomyelitis suum " from Denmark. Antisera against all 3 types of human polio virus failed to neutralize it.

A strain of Teschen virus from Germany was neutralized by Talfan antisera.

The virus of Talfan disease thus appears to be closely related to or identical with that of Teschen disease.

## REFERENCES

HARDING, J. D. J., DONE, J. T. AND KERSHAW, G. F.—(1957) *Vet. Rec.*, **69**, 824.

BALDUCCI, D., ZAIMAN, E. AND TYRRELL, D. A. J.—(1956) *Brit. J. exp. Path.*, **37**, 205.

BENDIXEN, H. C. AND SJOLTE, J. P.—(1955) *Nord. Vet. Med.*, **7**, 97.

DONE, J. T.—(1957) Proc. 75th Ann. Congr. B.V.A., Cambridge, 1957. *Vet. Rec.*, **69**, 1341.

LARSKI, Z.—(1955) *Méd. vét. Varsovie*, **11**, 58.

MAYR, A.—(1957) *Zbl. Bakt.* (1 *Abt. Orig.*), **168**, 350.

*Idem* AND SCHWOBEL, W.—(1956) *Mh. prakt. Tierheilk.*, **8**, 49.—(1957) *Zbl. Bakt.* (1 *Abt. Orig.*), **168**, 336.

# FURTHER STUDIES ON INTERFERON

D. C. BURKE AND A. ISAACS

*From the National Institute for Medical Research, Mill Hill, N.W.7*

Received for publication November 7, 1957

IN a series of studies of the viral interference reaction it was found that a product of the interference phenomenon, called interferon, was formed and was itself capable of inducing interference (Isaacs and Lindenmann, 1957). Interferon appears to be a protein which is taken up by chorioallantoic membranes rendering them unable to support viral multiplication (Lindenmann, Burke and Isaacs, 1957). In this paper we report further attempts to characterize interferon and to describe the conditions for its production.

## MATERIALS AND METHODS

These were generally those of the previous paper (Lindenmann *et al.*, 1957) except that ultra-violet irradiated (u.v.) virus was used routinely for the preparation of interferon. The virus strains used were the classical Melbourne and PR8 strains of influenza A, the influenza strain A/Persia/2/52 which produces abundant filaments, and the haemagglutinating virus of Japan or Sendai virus (Kuroya, Ishida and Shiratori, 1953). The latter strain was used whenever only small amounts of interferon were present because of its sensitivity to interferon. Incomplete influenza virus was prepared by the technique of von Magnus (1951) in which three passages of undiluted infective allantoic fluid were made in the allantoic cavity. Third passage virus was found to have a ratio of infectivity/haemagglutinin titre of $< 0{\cdot}1$ per cent of standard virus.

Enzymes: pepsin, a crystalline preparation of porcine origin from Armour & Co. (lot 12655) and ribonuclease, a crystalline preparation from Worthington Biochemical Co. (lot A546). Phosphorus was determined by a micro modification of the method of Allen (1940).

## RESULTS

### *Dosage of heat and u.v.-inactivated virus.*

It has been previously observed (Lindenmann *et al.*, 1957) that u.v.-inactivated virus produces more interferon than heat-inactivated virus and an experiment was designed to examine quantitatively their relative efficiency in producing interferon and the effect of dosage with the two types of virus used. Heat and u.v.-inactivated virus were adjusted to the same haemagglutinin titre (1200) and the different doses, made up to 50 ml. with buffer, shaken for 3 hr. at 37° with 5 whole chorioallantoic membranes in 1 litre Roux bottles. The membranes were washed, fresh buffer was introduced and they were then shaken overnight to produce interferon in the usual way. Titration of the fluids gave the results shown in Fig. 1 in which the dose of virus used is plotted against the yield of interferon produced.

It can be seen that u.v.-inactivated virus is much more efficient in producing interferon than an equivalent amount of heat-inactivated virus. Since it was not clear whether maximal production of interferon had been reached at a dose of

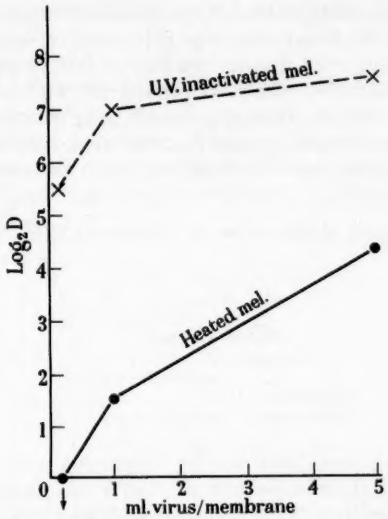



FIG. 1.—Production of interferon by varying doses of heat and u.v.-inactivated virus.

5 ml. of heated virus per membrane, the amount of interferon produced by a wider range of heat-inactivated virus was determined. The results are shown in Table I.

TABLE I.—*Production of Interferon by Heat-inactivated Influenza Virus*

| | | Material tested | Virus yield after challenge: $-\log_2$ HA titre* | Virus yield after challenge: Per cent of control group |
|---|---|---|---|---|
| Group No. | 1 | 1 ml. heated MEL/membrane | 6·4 | 50 |
| | 2 | 2 ,, ,, ,, ,, | 3·0 | 4·7 |
| | 3 | 4 ,, ,, ,, ,, | 2·5 | 3·3 |
| | 4 | 8 ,, ,, ,, ,, | 1·1 | 1·4 |
| | 5 | Buffer | 7·4 | 100 |

* Expressed as geometric mean titre of dilution end-point ($-\log_2$) in this and in the following tables.

This suggests that heat-inactivated virus is capable of producing as much interferon as u.v.-inactivated virus, but at a much higher dosage per membrane.

*Attempted preparation of interferon directly from inactivated virus*

An attempt was made to determine whether interferon was a part of the infecting virus or not, by sonic disruption of influenza virus filaments and examination of the fluid for interfering activity. A/Persia/2/52 allantoic fluid was centrifuged at 1000 *g* for 30 min. and the deposit suspended in one-tenth the volume of saline in order to concentrate filaments. The fluid was divided into three portions; one was irradiated with u.v. light for 2 min. under the usual conditions, the second

was irradiated as before and followed by ultra-sonic treatment (six half-minute bursts of 350 k.c.), while the third was given ultra-sonic treatment only. The latter two portions were dialysed with stirring against an 0·05 M pH 1 buffer for 2 hours to destroy intact virus and then dialysed against pH 7·4 buffer overnight. Interferon is known to be stable on treatment at pH 1 in this way.

The fluids were then assayed for their interfering activity (in the usual way except that Sendai virus was used for challenge) with the result shown in Table II.

TABLE II.—*Attempted Preparation of Interferon from Inactivated Virus*

| | | Material tested | Virus yield after challenge: $-\log_2$ HA titre | Virus yield after challenge: Per cent of control group |
|---|---|---|---|---|
| Group No. | 1 | u.v.-irradiated | 1 | 12 |
| | 2 | " " , sonics and pH 1 | 5·3 | 158 |
| | 3 | Sonics and pH 1 | 4·3 | 113 |
| | 4 | Buffer control | 4·1 | 100 |

If any interferon had been liberated by treatment with ultra-sonics it would have been stable at pH 1 (Lindenmann *et al.*, 1957) and should have been detected in group No. 2. There is thus no evidence from this experiment to suggest that interferon is part of the inactivated virus.

*Production of interferon from live and u.v.-irradiated virus on the Chorion*

Heat-inactivated virus has been shown to produce interferon when inoculated on to the chorionic surface (Lindenmann *et al.*, 1957) and it was of interest to see whether live virus would produce interferon in this system. The first experiments showed that barely significant amounts of interferon were being produced and so the following experiment was set up. Freshly harvested PR8 allantoic fluid was adsorbed on to 1 per cent red cells and eluted into half the volume of saline. The eluate (haemagglutinin titre 2400–4800) was irradiated with u.v. light in 3 ml. portions for 0·5, 1, 2, 4 and 8 min. and infectivities were determined on these fluids and on the unirradiated eluate. Groups of 11-day eggs were inoculated on the dropped chorionic surface with 0·5 ml. of each of the irradiated fluids and of unirradiated eluate (4 eggs per group). After incubation at 37° for 90 min. 0·1 ml. of PR8 serum (heated at 56° for 30 min.) was added to each egg. After a further 30 min. incubation the eggs were opened, the chorions removed and washed, and the pooled membranes from each group incubated at 37° on the roller drum overnight with 2 ml. of buffer and 0·1 ml. of antiserum. Under these conditions no haemagglutinin was produced. The fluids were diluted with buffer to 6 ml. and tested for the presence of interferon using Sendai as challenge virus. Serological tests showed that only Sendai virus was produced. The results of two experiments are shown in Fig. 2 as a plot of time of irradiation against interferon yield; in the first experiment the irradiated materials had no infectivity while in the second, some of the fluids showed a very low infectivity ($< 10^{-1}$) and there was no clear relationship apparent between loss of infectivity and production of interferon.

These results show that short periods of irradiation led to maximal production of interferon but that on further irradiation, or with unirradiated material, very

little interferon was produced. Two other experiments gave essentially similar results.

*Production of interferon by complete and incomplete virus on the chorion*

Since interferon was only produced after loss of infectivity it was decided to compare the efficiency of complete and incomplete virus in producing interferon on the chorion. It is known that incomplete virus is capable of inducing interference provided sufficient time is allowed before challenge (von Magnus, 1954).

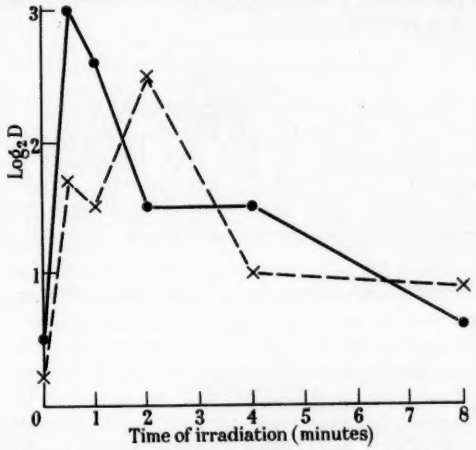



FIG. 2.—Production of interferon on the chick chorion by virus irradiated with u.v. for varying times. First experiment ———, second experiment .....

Accordingly, incomplete (third undiluted passage) and complete (diluted passage) PR8 virus were adjusted to the same haemagglutinin titre and 1 ml. portions inoculated on to the dropped chorion of 11-day eggs (4 per group). The eggs were treated as described in the previous experiment with the results shown in Table III.

TABLE III.—*Production of Interferon by Complete and Incomplete Virus on the Chick Chorion*

| Materials tested | I.D.$_{50}$/0·05 ml. | Virus yield after challenge | |
|---|---|---|---|
| | | — $\log_2$ HA titre | Per cent of control |
| Complete PR8 | $10^{9.5}$ | 2·3 | 47 |
| Incomplete PR8 | $10^{5.8}$ | 0·1 | 11 |
| Buffer control | .. | 3·4 | 100 |

Another experiment gave an essentially similar result.

*Attempted preparation of an interferon competitor*

One explanation of the failure of live virus to produce interferon when inoculated on to the chorion was that damaged virus produced interferon which was

a competitor with a normal component of the multiplication cycle of live virus. To test this, 1 ml. portions of infective PR8 virus were inoculated on to the dropped chorion of 11-day eggs and then treated as in the last two experiments. Both the fluids and a membrane extract (eight membranes ground with glass powder and 5 ml. of buffer followed by light centrifugation) were tested for the presence of a possible interferon competitor by mixing with a standard interferon preparation and testing for interfering activity in the usual way. No inhibitor of interferon activity was found in such membrane fluids or extracts. However, we cannot rule out the possibility that an inhibitor of interferon activity is produced in the membrane and is rapidly used up.

*Miscellaneous*

Confirmation of the protein nature of interferon was obtained by digestion with pepsin. Interferon was adjusted to pH 2 by dialysis against a 0·05 M potassium chloride/hydrochloric acid buffer and half was digested with crystalline pepsin (final concentration 0·001 per cent) at 37° for one hour, while the remainder was incubated at 37° as a control. This was followed by dialysis against pH 7·4 buffer and testing for interferon with the results shown in Table IV.

TABLE IV.—*Action of Pepsin on Interferon*

| | | Material tested | Virus yield after challenge: $-\log_2$ HA titre | Virus yield after challenge: Per cent of control |
|---|---|---|---|---|
| Group No. | 1 | Interferon | 2·1 | 0·9 |
| | 2 | Buffer + Pepsin | 8·9 | 100 |
| | 3 | Interferon + Pepsin | 9·1 | 113 |

Interferon was not affected by prolonged digestion with ribonuclease (in saline at a final concentation of 0·001 per cent for 24 hr. at 37°) or by addition of sodium iodoacetate (at a final concentration of 0·01 M for 30 min. at room temperature), but it was completely destroyed by shaking for 5 min. with an equal volume of amyl alcohol/chloroform (1 : 2). It was only slowly inactivated by irradiation with u.v. light, (maximal emission at 2537 Å) as shown in Table V.

TABLE V.—*Effect of Ultra-Violet Irradiation on Interferon*

| | | Material tested | Virus yield after challenge: $-\log_2$ HA titre | Virus yield after challenge: Per cent of control |
|---|---|---|---|---|
| Group No. | 1 | Interferon | 1·3 | 1 |
| | 2 | " irradiated 5 min. | 2·2 | 1·8 |
| | 3 | " " 10 " | 2·9 | 2·9 |
| | 4 | " " 20 " | 3·8 | 5·4 |
| | 5 | Buffer | 8·0 | 100 |

This behaviour is not inconsistent with the formulation of interferon as protein or partially protein.

In a previous paper (Isaacs, Lindenmann and Valentine, 1957) the heat lability of interferon was advanced as an argument against its being a ribonuclease. Mr. N. W. Pirie has pointed out to us that not all ribonucleases are heat stable,

and to settle this point a direct assay of an interferon preparation for ribonuclease activity by a standard procedure was carried out (Colowick and Kaplan, 1955). A typical interferon preparation contained less than the equivalent of 0·15 $\mu$g. of pancreatic ribonuclease per ml. and this amount is insufficient to induce significant interference in the system used. Interferon would have to be at least thirteen hundred times more active than pancreatic ribonuclease on a weight basis to account for the degree of interference found.

## DISCUSSION

From previous work it has not been possible to decide whether interferon is part of the inactivated virus used in its preparation, or whether it is newly synthesized in the membrane. The time of appearance of interferon and the presence of an eclipse phase (Isaacs and Lindenmann, 1957) favoured the second interpretation and this is supported by the effect of different dosages of heat and u.v.-inactivated virus on the yield of interferon. The flattening of the curve obtained with u.v.-inactivated virus (Fig. 1) could be due either to the inability of the cells to handle more than a limited number of viral particles or to a limited ability to synthesize interferon. However, the results with heat-inactivated virus make it clear that the number of viral particles is not the limiting factor. This suggests that interferon is newly synthesized in the membrane, but a firm decision on this point may require the use of radioactive labelled virus and membranes. There is some evidence that the saturation effect is a temporary one. We have already found that membranes given a second inoculum of inactivated virus produced a second crop of interferon (Lindenmann *et al.*, 1957) and more recently we found that third and fourth crops of equal potency can be obtained in the same way.

The results obtained with u.v.-irradiated virus on the chorion suggest that the virus particles must be slightly damaged before interferon can be produced. It is known (Powell and Setlow, 1956) that the rapid loss of viral infectivity on u.v.-irradiation is primarily due to damage of the viral nucleic acid and although there does not seem to be any relationship between loss of infectivity and interferon production, the short times of irradiation at 2537 Å necessary to obtain maximal yields of interferon suggest that the damage discussed above is in the nucleic acid or nucleoprotein of the virus. This idea is supported by the results obtained with incomplete virus since the most significant difference between incomplete and complete virus is the lower nucleic acid content of incomplete virus (Ada and Perry, 1956). The fact that incomplete virus has been found to be a much more efficient producer of interferon than complete virus again suggests that it is viral particles with damaged nucleic acid or nucleoprotein that initiate interferon production. However, excessive damage with u.v.-irradiation greatly reduced the amount of interferon produced. This suggests that interferon is an analogue of some intermediate normally synthesized during the growth of virus. If there is such an intermediate, we have until now been unable to demonstrate it.

## SUMMARY

The production of interferon by varying amounts of heat and u.v.-inactivated virus has been examined. The results suggest that interferon is newly synthesized in the membrane, rather than part of the inactivated virus.

Interferon was produced by u.v.-inactivated virus on the chick chorion. Live influenza virus grown under similar conditions or heavily irradiated virus did not produce interferon, whereas incomplete virus produced good yields. The findings suggest that interferon is produced by virus in which the nucleic acid has been slightly, but not heavily, damaged.

We should like to thank Dr. C. H. Andrewes, F.R.S., for his advice and criticism and Mr. V. G. Law and Miss V. Coker for their technical help.

Recently Dr. F. Fulton pointed out to us that in estimating the size of interferon by filtration (Isaacs, Lindenmann and Valentine, 1957) we had not diluted interferon in broth. We therefore filtered some interferon, diluted in buffer, through a 0·5$\mu$ A.P.D. membrane and then filtered some of this material diluted in broth through the same membrane. Interferon activity was absent from the first filtrate but present in the second. In subsequent experiments we found that when interferon was diluted in broth and filtered through a collodion membrane of A.P.D. 0·048$\mu$ some interferon activity passed through the membrane. Thus the earlier filtration measurements were in error and this subject is being further investigated.

## REFERENCES

Ada, G. L. and Perry, B. T.—(1956) *J. gen. Microbiol.*, **14**, 623.
Allen, R. J. L.—(1940) *Biochem. J.*, **34**, 858.
Colowick, S. P. and Kaplan, N. O.—(1955) 'Methods in Enzymology'. New York (Academic Press), **2**, 427.
Isaacs, A. and Lindenmann, J.—(1957) *Proc. Roy. Soc. B*, **147**, 258.
*Iidem* and Valentine, R. C.—(1957) *Ibid.*, **147**, 268.
Kuroya, M., Ishida, N. and Shiratori, T.—(1953) *Yokohama med. Bull.*, **4**, 217.
Lindenmann, J., Burke, D. C. and Isaacs, A.—(1957) *Brit. J. exp. Path.*, **38**, 551.
Powell, W. F. and Setlow, R. B.—(1956) *Virology*, **2**, 337.
von Magnus, P.—(1951) *Acta. path., microbiol. scand.*, **28**, 278.—(1954) 'Advances in Virus Research'. New York (Academic Press), **2**, 59.

# THE EFFECT OF OXYTETRACYCLINE AND NEOMYCIN ON AMMONIA LEVELS IN THE MESENTERIC VEIN AND ON THE CAECAL FLORA IN RABBITS*

B. RUEBNER† AND JANET R. McLAREN

*From the Departments of Pathology and Medicine, Postgraduate Medical School of London, Ducane Road, London, W.12*

Received for publication November 12, 1957

LARGE quantities of ammonia are normally present in portal blood and are metabolized to urea in the liver (Bollman and Mann, 1930). Parnas and Klisiecki (1926) found that in the rabbit the ammonia level was approximately fifty times higher in the veins draining the lower small intestine, caecum and upper large intestine than in peripheral blood. Cholopoff (1927) obtained similar results in the dog and concluded from his experiments that most of the ammonia in the body was produced by bacterial action in the large intestine and appendix. Phear and Ruebner (1956) found that all the common intestinal bacteria except possibly some lactobacilli produced ammonia *in vitro*.

A raised ammonia level in the peripheral circulation may have toxic effects. Bollman and Mann (1930), for example, found that ammonium salts were toxic to dogs with a porta-caval shunt (Eck-fistula). Neurological abnormalities were also produced in such animals by feeding a high protein diet (Monguio and Krause, 1934). A similar correlation between high peripheral blood ammonia levels and neurological symptoms was observed in patients with a porta-caval shunt (McDermott and Adams, 1954) and in patients with cirrhosis of the liver (Sherlock, Summerskill, White and Phear, 1954). White, Phear, Summerskill and Sherlock (1955) showed that ammonium salts were toxic to patients with cirrhosis of the liver. It is therefore generally believed that ammonia produced in the intestine may be an aetiological factor in hepatic coma. Farquhar, Stokes, Whitlock, Bluemle and and Gambescia (1950) found that chlortetracycline benefited patients suffering from this condition. Attempts to lower the peripheral vein ammonia by antibacterial agents have been made both in Eck-fistula dogs and in patients with cirrhosis or a porta-caval shunt. Sulphonamides appeared to be effective in a patient with a porta-caval shunt (McDermott and Adams, 1954) as did neomycin in patients with cirrhosis of the liver (Fisher and Faloon, 1957). Mann, Bollman, Huizenga, Farrar and Grindlay (1954) however, found no demonstrable effect on the peripheral blood ammonia in Eck-fistula dogs treated with oxytetracycline or neomycin. Because of these contradictory results the effect of oral antibacterial agents on the ammonia levels in the veins draining the intestinal tract was investigated. Silen, Harper, Maudsley and Weirich (1955) found in dogs that neomycin lowered the ammonia level to less than half the control value, whereas tetracycline was

* This work was supported by grants from the Medical Research Council.

† Present address: Department of Pathology, Dalhousie University, Halifax, Nova Scotia, Canada.

ineffective. The present study concerns a similar investigation of the caecal flora of rabbits. The caecum is the principal source of ammonia in these animals (Parnas and Klisiecki, 1926).

## MATERIAL AND METHODS

### *Experimental animals*

Rabbits weighing 1·5–2·5 kg. were used. Eight control animals received no antibiotics and a further eight were given 1 g. of oxytetracycline orally in four divided doses during the 48 hr. preceding the experiment. This period was adopted as it seemed optimal for the effect of intestinal antibiotics (Campos, Pontes, Hoenen and Kusminski, 1955). Three other rabbits were given a total dose of 2 g. of neomycin sulphate (equivalent to 1·4 g. of neomycin) similarly divided into four doses. The animals were fed on MRC diet No. 18 containing: protein 16·5 per cent, carbohydrate 33·7 per cent, and fat 4·6 per cent. Fresh vegetables and water were given daily. No loss of appetite was noted in any of the animals receiving antibiotics.

Each animal was anaesthetized by intravenous injection of approximately 1 ml. of 0·5 per cent Nembutal. At laparotomy a sample of caecal contents was aspirated into a sterile syringe through a wide-bore needle, usually after prior injection of saline containing 2 per cent broth, so that an adequate specimen could be obtained.

### *Bacteriological methods*

The bacterial flora of the caecum was investigated by a semi-quantitative method similar to that of Cregan and Hayward (1953). Four media were used: 5 per cent horse blood agar, heated blood agar, McConkey's medium and Sabouraud's medium. A standard loopful (0·01 ml.) of the caecal aspirate was spread over four successive areas of each and incubated at 37° (Sabouraud's at 22°) for 48 hr. A further blood agar plate and a heated blood agar plate were similarly treated but incubated anaerobically with 5 per cent $CO_2$ in a McIntosh and Fildes jar. Growth was assessed as +, ++, +++ or ++++ according to the number of areas showing growth after incubation.

### *Estimation of blood ammonia*

Blood was obtained from the mesenteric vein draining the caecum (which had a very constant course in different animals) and the blood ammonia was estimated by the micro-diffusion method of Conway (1950).

At the end of the experiment the animals were killed by an overdose of Nembutal.

## RESULTS

At laparotomy gaseous distension of the caecum was noted in those rabbits which had been given oxytetracycline but not in any of those treated by neomycin or in the controls.

The mean ammonia level in the mesenteric vein of oxytetracycline-treated animals was only about a third of that found in the control animals (see Table I) This fall was statistically significant ($t = 5{\cdot}1$ ; $p < 0{\cdot}001$). No significant change, however, was observed in animals given neomycin.

Gram films of the faeces of all three groups of animals showed mainly small Gram negative bacilli and a few large Gram positive spore bearing bacilli. By far the most predominant organisms found in cultures from the control animals were Gram negative anaerobic bacilli of the *Bacteroides* group. The sole constant bacteriological change in the oxytetracycline-treated group was the elimination of these organisms. There was no reduction of *Bacteroides* or any other significant effect in the neomycin-treated animals.

TABLE I.—*The Relationship of Ammonia Nitrogen (μg./ml.) in the Caecal Mesenteric Vein to the Bacterial Growth Obtained from Caecal Aspirates*

Control rabbits

| Animal No. | Ammonia N | Bacterial growth: *Bacteroides* | Bacterial growth: Gram + aerobic spore bearer | Bacterial growth: *Bact. coli* |
|---|---|---|---|---|
| 1 | 14·7 | ++++ | − | + |
| 2 | 12·3 | ++++ | − | − |
| 3 | 22·0 | ++++ | − | − |
| 4 | 27·0 | ++++ | + | − |
| 5 | 20·0 | ++++ | + | − |
| 6 | 10·2 | ++++ | − | − |
| 7 | 15·6 | ++++ | − | + |
| 8 | 15·3 | ++++ | + | − |
| Mean | 17·1 (S.D. = 5·6) | | | |

Rabbits given oxytetracycline

| Animal No. | Ammonia N | Bacterial growth: *Bacteroides* | Bacterial growth: Gram + aerobic spore bearer | Bacterial growth: *Ps. pyocyanea* |
|---|---|---|---|---|
| 9 | 8 | − | + | − |
| 10 | 5·5 | − | − | − |
| 11 | 12·0 | − | + | + |
| 12 | 7·3 | − | + | − |
| 13 | 7·7 | − | − | − |
| 14 | 4·4 | − | − | − |
| 15 | 6·8 | − | − | − |
| 16 | 4·5 | − | − | − |
| Mean | 6·6 (S.D. = 3·3) | | | |

Rabbits given neomycin

| Animal No. | Ammonia N | Bacterial growth: *Bacteroides* | Bacterial growth: Gram + aerobic spore bearer | Bacterial growth: *Cl. sporogenes* |
|---|---|---|---|---|
| 17 | 16·0 | ++++ | − | + |
| 18 | 16·0 | ++++ | − | − |
| 19 | 15·0 | ++++ | − | − |
| Mean | 15·7 | | | |

## DISCUSSION

Distension of the caecum after tetracycline therapy was previously observed in rats by Reynell (1953). Presumably this effect is in some way associated with intestinal sterilization.

In agreement with the work of Silen *et al.* (1955) in dogs the present investigation shows that in rabbits intestinal antibiotics can lower the ammonia level in the veins draining the intestine. In their experiments, however, neomycin was found to be effective but not tetracycline whereas in the present experiments oxytetracycline and not neomycin lowered the blood ammonia level. This discrepancy may be due to a difference in the intestinal flora between the dog and the rabbit. Silen *et al.* (1955) found coliform organisms, enterococci and clostridia

in the intestinal contents of their dogs while the only organisms isolated in large numbers from the rabbit's caecum in the present work were strains of *Bacteroides*. There are few previous references to the intestinal flora of the rabbit (Frank, Hadeler and Harder, 1951). The *Bacteroides* were sensitive to the tetracyclines but not to neomycin. The effect of the two antibiotics on *Bacteroides* agrees with the findings of most previous workers (Dearing and Heilman, 1950; Fog, 1954; Jawetz, 1956 and Gillespie and Guy, 1956) but not with those of Cohn and Longacre (1955–56). Gram stains showed little change both after neomycin and after two days of oxytetracycline therapy. Presumably the Gram negative organisms still seen after oxytetracycline were no longer viable.

While the mean ammonia level was lowered considerably by oxytetracycline a substantial quantity was nevertheless often present even after administration of this antibiotic. This ammonia may have been produced by intestinal organisms not isolated by the culture methods selected. This seems unlikely as special media for fungi as well as those for aerobic and anaerobic bacteria were used in this investigation. It appears more probable that this residual ammonia was derived from ingested food either directly or by digestion.

The contradictory effects of intestinal antibiotics on the peripheral ammonia levels noted by previous observers are probably due at least in part of the difficulty of significantly lowering the numbers of intestinal bacteria for more than 2 or 3 days. Recently Fisher and Faloon (1957) found that neomycin, at present probably the most effective intestinal antibiotic in man, did lower the peripheral blood ammonia level in patients with cirrhosis of the liver. It is suggested that these results could be improved by the addition of oxytetracycline or some other antibiotic effective against *Bacteroides*, one of the principal organisms in the human intestine.

SUMMARY

The mean ammonia level in the mesenteric vein of oxytetracycline-treated rabbits was only about a third of that found in control animals. No significant change occurred in animals given neomycin.

The predominant organisms isolated from caecal aspirates of the control animals were anaerobic bacilli of the *Bacteroides* group. These were eliminated in the oxytetracycline group but not in rabbits given neomycin.

It is concluded that the production of ammonia, which is toxic to patients with cirrhosis of the liver, can be lowered but not abolished by effective intestinal antibiotics.

The importance of ammonia production by anaerobic intestinal organisms, particularly the *Bacteroides* group, is emphasized.

## REFERENCES

Bollman, J. C. and Mann, F. C.—(1930) *Amer. J. Physiol.*, **92**, 92.

Campos, J. V., Pontes, F. J., Hoenen, W. and Kusminski, N.—(1955) *J. Lab. clin. Med.*, **45**, 455.

Cohn, I. and Longacre, A. B.—(1955–56) Antibiotics Annual Medical Encyclopaedia Inc., New York, p. 105.

Cholopoff, A. D.—(1927) *Pflüg. Arch. ges. Physiol.*, **218**, 670.

Conway, E. J.—(1950) 'Microdiffusion Analysis and Volumetric Error'. Third ed. London (Crosby Lockwood).

CREGAN, J. AND HAYWARD, N. J.—(1953) *Brit. med. J.*, i, 1356.
DEARING, W. H. AND HEILMAN, F. R.—(1950) *Proc. Mayo Clin.*, **25**, 87.
FARQUHAR, J. D., STOKES, J., WHITLOCK, C. M., BLUEMLE, W. AND GAMBESCIA, J. M. —(1950) *Amer. J. med. Sci.*, **220**, 166.
FISHER, C. J. AND FALOON, W. W.—(1957) *New Eng. J. Med.*, **256**, 1030.
FOG, C. V. M.—(1954) *Acta. chir. scand.*, **107**, 291.
FRANK, I., HADELER, U. AND HARDER, W.—(1951) *Pflüg. Arch. ges. Physiol.*, **253**, 385.
GILLESPIE, W. A. AND GUY, J.—(1956) *Lancet*, i, 1039.
JAWETZ, E.—(1956) ' Polymyxin, Neomycin and Bacitracin ', Antibiotics Monograph No. 5, New York.
MANN, J. D., BOLLMAN, J. L., HUIZENGA, K. A., FARRAR, T. AND GRINDLAY, J. H.— (1954) *Gastroenterology*, **27**, 399.
MCDERMOTT, W. V. AND ADAMS, R. D.—(1954) *J. clin. Invest.*, **33**, 1.
MONGUIO, J. AND KRAUSE, F.—(1934) *Klin. Wschr.*, **13**, 1142.
PARNAS, J. K. AND KLISIECKI, A.—(1926) *Biochem. Z.*, **173**, 224.
PHEAR, E. A. AND RUEBNER, B.—(1956) *Brit. J. exp. Path.*, **37**, 253.
REYNELL, P. C.—(1953) *J. Path. Bact.*, **66**, 47.
SHERLOCK, S., SUMMERSKILL, W. H. J., WHITE, L. P. AND PHEAR, E. A.—(1954) *Lancet*, ii, 453.
SILEN, W., HARPER, H. A., MAWDSLEY, D. L. AND WEIRICH, W. L.—(1955) *Proc. Soc. exp. Biol. Med.*, **88**, 138.
WHITE, L. P., PHEAR, E. A., SUMMERSKILL, W. H. J. AND SHERLOCK, S.—(1955) *J. clin. Invest.*, **34**, 158.

# A REACTION BETWEEN FRESH SERUM AND LIPOPOLYSACCHARIDES OF GRAM-NEGATIVE BACTERIA

D. ROWLEY, W. ALI AND C. R. JENKIN

*From the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2*

Received for publication September 25, 1957

IT has been shown that in the case of some Gram-negative organisms there is a correlation between the virulence of the strain for mice and the resistance of that strain to the bactericidal activity of fresh complement-containing serum (Maaloe, 1948; Rowley, 1954). It seems reasonable that the sensivity of any strain to the serum properdin-complement system might depend on the amount or availability of the bacterial substrate of this reaction. On the basis of a substrate-competition test, it appeared possible that the lipopolysaccharide fraction occurring in the cell walls of Gram-negative bacteria might constitute the bacterial substrate for this serum bactericidal reaction (Rowley, 1956). The question arises, if this is the substrate, what is the nature of its reaction with serum? This paper describes in detail the preparation of $P^{32}$-labelled lipopolysaccharide and the dephosphorylating reaction which occurs when this material is incubated with fresh serum (Rowley, Howard, and Jenkin, 1956).

## MATERIAL AND METHODS

*Strains.*—The following strains were used: *Bact. coli*, 2380, 2206, 145, K12 (Rowley, 1954) Co. 18 (Kroger), Lilly (Wardlaw). *Salm. paratyphi B.* $B_IS$, $B_IR$, $B_{II}S$, $B_{II}R$, from Dr. E. Kroger.

In vitro *bactericidal tests.*—These were performed according to the method previously described (Rowley, 1956).

*Alkaline phosphatase* determinations were carried out according to the methods of King and Armstrong as described by Harrison (1947).

*Properdin.*—One sample of bovine origin was obtained from Prof. L. Pillemer, others were prepared from human serum and assayed using the methods of Pillemer, Blum, Lepow, Ross, Todd and Wardlaw (1954) by Dr. A. C. Wardlaw of this Institute.

*$P^{32}$ lipopolysaccharides* ($P^{32}$-LP).—These were prepared from bacteria grown on a medium of the following composition: "Difco" casamino acids 10 g., Tris-(hydroxy-methyl)-amino methane 20 g., ammonium sulphate 10 g., sodium citrate 5 g., glucose 1 g., agar 20 g. in 1 litre of water at pH 7·5. The 20 g. agar and 800 ml. water were autoclaved for 15 min. at 10 lb./sq. in. in a 1 litre bottle; while still hot 100 ml. sterile 10 per cent Difco casamino acid (phosphate free) and 100 ml. sterile 20 per cent Tris Buffer salts solution were added aseptically followed by 10 mc. of $P^{32}$ in the form of 1 ml. carrier-free sodium phosphate solution. The medium was then dispensed in 100 ml. amounts in 1 litre flat bottles and allowed to set in a thin layer. The bottles of medium were inoculated with a washed suspension of bacteria and incubated at 37° for 48 hr., before harvesting in saline with the aid of glass beads. The suspension, containing approximately 0·5 g. dry weight of bacteria, was treated in a Müllard ultrasonic generater, type E7590A, for 30 min., after which time the turbidity was greatly reduced and most of the bacteria had been disrupted. The cell wall suspension was then extracted with 100 ml. 45 per cent phenol at 65° according to the method of Westphal, Luderitz and Bister (1952). The $P^{32}$-LP was obtained from the aqueous layer by precipitation with 5 vol. of alcohol and purified by deposition from aqueous solution in the Spinco ultra-

centrifuge at 40,000 r.p.m. for 2 hr. Examination in the Beckman spectrophotometer showed little or no absorption in the region of 260–280 mμ indicating contamination with less than 0·1 per cent nucleic acid. The initial specific activity of the lipopolysaccharide preparations was about 2600 counts/μg./min.

*Assay of radioactivity.*—For this purpose we used a thin mica end-window Geiger counter, installed in a Nuclear-Chicago Automatic sample changer type C110A, with an automatic printing timer C111 coupled to the model 183 scaling unit, to record the results. This apparatus was modified by the insertion of a G.E.C. helium filled thin window Geiger tube which increased the sensitivity of the machine threefold.

Samples of 0·1 ml. were pipetted from the dialysers on to stainless steel sample pans and one drop of 1 per cent gelatine solution added to each sample before drying under an infra-red lamp. These were then assayed by determining the time taken to achieve a total count of 640, giving a probable error of approximately ± 3 per cent. Each determination was corrected by subtracting the " background " count of 10–12 counts/min.

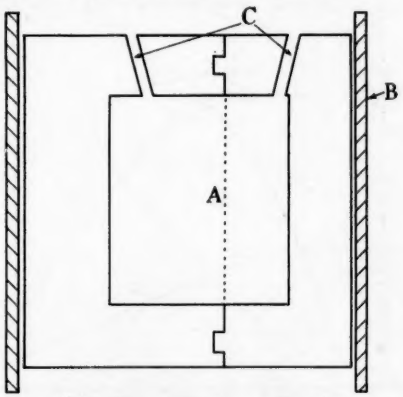



FIG. 1.—Section through perspex dialyser.

A.—Cellophane membrane.
B.—Metal clamping plates.
C.—Sampling apertures.

*Reaction of serum with lipopolysaccharides.*—The principle of the methods was to look for small molecular $P^{32}$-containing fragments which might be liberated from the high molecular non-dialysable $P^{32}$ by the action of serum. Most of the experiments were carried out in the following way :

One ml. of serum dilutions in veronal buffer or other non-phosphate containing buffers, were pipetted into test tubes and placed in a water bath at 37°. After a few minutes $P^{32}$-LP was added, usually in a volume of 0·02 ml. containing 10–15 μg. of lipopolysaccharide. The tubes were incubated for a further 30 min. and then cooled rapidly to 0° to stop the reaction. The whole of the material in each tube was then transferred to the small compartment of a perspex dialyser as shown in Fig. 1 and allowed to dialyse over-night in the refrigerator against the saline which filled the larger compartment. Aliquots of 0·1 ml. were then taken from the saline-containing compartment for radioactive assay. The split $P^{32}$ was calculated from this on the assumption that the dialysable material was equilibrated throughout the 4 ml. of the dialyser and is expressed as a percentage of the total $P^{32}$ in the lipopolysaccharide. In each experiment a control tube was set up containing $P^{32}$-LP incubated with the buffer diluent only.

*Preparation of alkaline phosphatase from calves intestines.*—The procedure described by Morton (1954) was followed up to stage 4. The final material obtained by us had an activity of 650 King-Armstrong (K.A.) units/mg.

*Veronal buffer and phenol-phosphate solutions* were those used in the King-Armstrong alkaline phosphatase determinations.

## RESULTS

As much as 25 per cent of the total phosphorus of the non-dialysable lipopolysaccharide was liberated in the form of dialysable $P^{32}$-containing material by incubation with fresh rat serum. The factors in the serum responsible for this effect were heat-labile (Table I). Fig. 2 shows that the reaction has a pH optimum between 8 and 9 and a temperature optimum of around 37°. The liberated dialysable $P^{32}$ was co-precipitated with magnesium ammonium phosphate and with ammonium phosphomolybdate and so may be assumed to consist of phosphate ions. Furthermore removal of divalent cations from the serum by shaking with

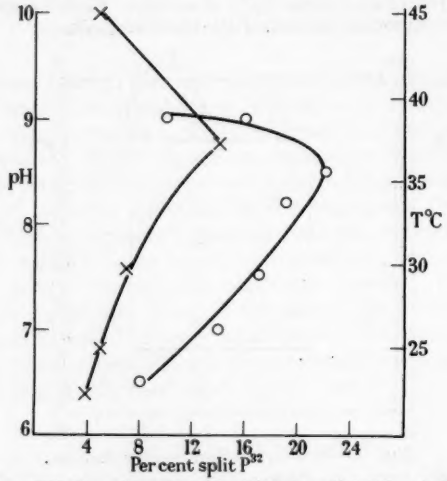



FIG. 2.—Reaction between $P^{32}$ lipopolysaccharide of *Bact. coli* 2380 and diluted rat serum at different temperatures and pH.
×—× Temperature, rat serum 1/20.
○—○ pH, rat serum 1/5.

I.R.C. 50 resin followed by chelation of any remaining divalent cations by addition of 0·0005 M ethylene diamine tetra-acetate decreased the amount of $P^{32}$ liberated from 21 per cent to 7 per cent. These properties, particularly the pH optimum, the relative requirements for divalent cations and the liberation of inorganic phosphate ions, suggest strongly that this reaction is due to an alkaline phosphatase in the serum. Table I also lists results obtained with sera from different animals.

TABLE I.—*Liberation of Dialysable $P^{32}$ from $P^{32}$-LP of* Bact. coli 2380 *by Action of Serum* (10 *μg. LP/ml. Serum Dilution*)

| Conditions | Percentage split $P^{32}$ |
|---|---|
| Rat serum—diluted 1 : 2 | 21 |
| Rat serum 1 : 2 treated with IRC50 + 0·0005 M E.D.T.A. | 7 |
| Rat serum 1 : 2 heated 56° for 20 min. | <1 |
| Undiluted rat serum | 18–21 |
| ,, mouse serum | 14–18 |
| ,, human serum | 14–16 |
| ,, guinea-pig serum | 10–12 |
| ,, rabbit serum | 8 |

*Effect of alkaline phosphatase from other sources on the lipopolysaccharide*

Partially purified alkaline phosphatase from calves' intestines with an activity of 650 K.A. units per mg. was tested for the ability to split phosphate from the lipopolysaccharides. When used at a strength of 1 mg./ml. with 20 $\mu$g. *Bact. coli* 2380 $P^{32}$-LP, 15 per cent of the available phosphate was liberated at pH 8·8, 2 per cent at pH 7 and none at pH 5.

The rate of the reaction was followed by incubation of $P^{32}$-LP with either rat serum diluted 1 : 5, i.e. 8 K.A. units, or alkaline phosphatase 0·25 mg/ml. i.e. 160 K.A. units, both in veronal buffer at pH 8·8. A series of tubes each containing 10 $\mu$g. $P^{32}$-LP and 1 ml. serum or enzyme dilution, was incubated at 37° and tubes removed at carefully timed intervals, cooled rapidly to 0° and transferred to one compartment of the dialyser cells to dialyse against 3 ml. saline over-

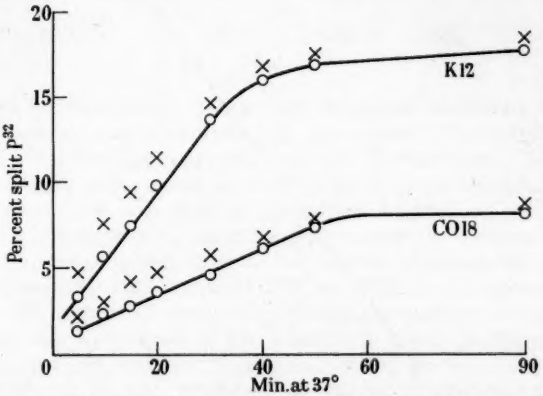



FIG. 3.—Rate of reaction of $P^{32}$-lipopolysaccharide from *Bact. coli* strains K.12 and Co.18.
○ Rat serum 1/5, 8 K.A. units/ml.
× Alkaline phosphatase, 160 K.A. units/ml.

night at 0°–2°. The radioactivity determined in the large compartment was calculated as total dialysable $P^{32}$ assuming equilibration throughout both compartments of the cell. Fig. 3 shows that two lipopolysaccharides were hydrolysed to different degrees but that each reacted similarly with either diluted serum or alkaline phosphatase solution, both as regards amount of phosphate liberated and rate of the reaction.

Further confirmation of the similarity between the two reactions was obtained by adding alkaline phosphatase to those cells which had previously contained serum and *vice versa* ; in no case was there any further liberation of phosphate, showing that the same phosphate bonds were split by intestinal alkaline phosphatase as by the serum enzyme.

*Evidence that lipopolysaccharide splitting phosphatase is different from phenol phosphatase*

In the course of many experiments it became obvious that various samples of serum were capable of splitting lipopolysaccharides at extremely high dilutions ;

*i.e.*, with the $P^{32}$-LP from some bacterial strains it was found that rat serum was effective when diluted 1 in 1000. On the other hand, the partially purified alkaline phosphatase required about 1 K.A. unit to give any appreciable effect. In other words, the phenol phosphatase activity of serum and other enzyme preparations could not be equated with the ability of the material to hydrolyse $P^{32}$-LP. In Fig. 4 many experiments are recorded in which the percentage of liberated $P^{32}$ is plotted against the phenol phosphatase activity of the rat serum dilution or the dilution of impure alkaline phosphatase from calves' intestines. The activity of the rat serum appears to be 20 to 50-fold greater than would be expected on the basis of its phenol-phosphatase content.

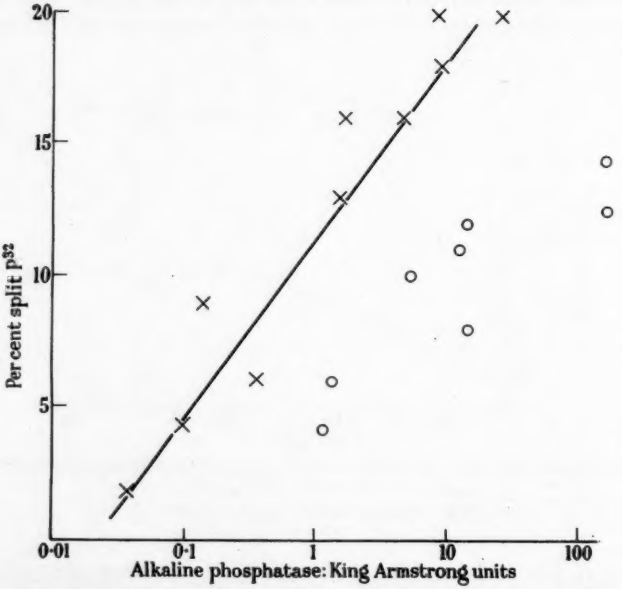



FIG. 4.—Reaction between $P^{32}$ lipopolysaccharide from *Bact. coli* 2206 and dilutions of rat serum or dilutions of intestinal alkaline phosphatase.

× Dilutions of rat serum.
○ Dilutions of intestinal alkaline phosphatase.

*Effect of phenol phosphate on lipopolysaccharide splitting by serum.*—Preliminary experiments using 200 $\mu$g. phenol phosphate in the presence of 10 $\mu$g. $P^{32}$-LP, showed that the hydrolytic action of rat serum diluted 1 : 5 was unimpaired by the phenol phosphate and a further experiment was set up in tubes as below (Table II).

The three tubes were incubated at 37° for 30 min. and then transferred to "cellophane" dialysis bags and 80,000 counts/min., *i.e.*, approximately 80 $\mu$g. $P^{32}$-LP from *Bact. coli* 2206 in 0·1 ml. added to each bag. This was then immersed in 10 ml. alkaline buffer and the whole incubated for 40 min., cooled to 0° and allowed to dialyse over-night. Samples were then withdrawn from the liquid

TABLE II.—*Effect of Phenol Phosphate on Splitting of $P^{32}$-LP by Serum*

| | Tube No. A (ml.) | B (ml.) | C (ml.) |
|---|---|---|---|
| Phenol phosphate solution | 1 | — | 1 |
| Saline | — | 1 | — |
| Veronal buffer, pH 8·8 | 1 | 1 | 1 |
| Serum | 0·1 | 0·1 | 0·1 (heated at 60° for 30 min.) |

Incubated at 37° for 30 min. 80 μg. $P^{32}$-LP added to each tube, incubated for a further 40 min., cooled to 0° and dialysed overnight against 10 ml. alkaline buffer.

| | | | |
|---|---|---|---|
| Amount of split, $P^{32}$ | 23% | 21% | 0% |

outside the bags for radioactive assay, and this was calculated in terms of the whole 12·2 ml. assuming equilibration. In the contents from tube A, there was 23 per cent split $P^{32}$, tube B, 21 per cent, and tube C, 0 per cent. A simple calculation reveals that there was a great excess of hydrolysable phosphate bonds in the form of phenol phosphate over that as lipopolysaccharide, *i.e.*, 950 μg. $PO_4$ in the 2 mg. of phenol phosphate and, since lipopolysaccharide contains approximately 2 per cent phosphorus of which only 25 per cent is hydrolysable by phosphatase there was approximately 1 μg. available $PO_4$ as $P^{32}$-LP. In spite of the fact that the phosphate ester groups of the phenol phosphate were in 1000-fold excess this had no effect on the hydrolysis of the phosphate ester groups of the lipopolysaccharide. The reverse situation was then studied by following the hydrolysis of phenol phosphate in the presence of excess lipopolysaccharide. Phenol phosphate solution 0·2 ml. was incubated with 0·2 ml. of rat serum, with and without the addition of 20 mg. purified lipopolysaccharide from *Bact. coli* 2206. After incubation for 30 min. the amount of phenol liberated was determined in the usual manner using the Folin and Ciocalteau reagent. In the tube containing lipopolysaccharide the amount of phenol liberated was approximately half of that in the other tube. The presence of 250 μg. of hydrolysable $PO_4$ in the lipopolysaccharide had competed with the 190 μg. $PO_4$ of the phenol phosphate, for the available enzyme.

*Hydrolysis of $P^{32}$-LP by properdin preparations*

Properdin has been shown to be involved in the serum bactericidal reaction and can be obtained free from the components of complement (Pillemer *et al.*, 1954). It seemed worthwhile to test this material for its hydrolytic activity. None of the preparations used, whether obtained from Professor Pillemer or from Dr. Wardlaw showed any King-Armstrong phenol phosphatase activity. Nevertheless, as seen in Table III all samples were active in releasing $P^{32}$ from $P^{32}$-LP and one preparation was still quite active when diluted 100-fold. These properdin preparations were all acknowledged to be impure, so it is not clear whether the substance responsible for the properdin activity is also the specific lipopolysaccharide-splitting alkaline phosphatase of serum.

TABLE III.—*Activity of Various Materials in Splitting $P^{32}$-LP from* Bact. coli 2380 *in Comparison with their King-Armstrong Phenol Phosphatase Activity*

| Sample | King-Armstrong alkaline phosphatase at strength used | Percentage split $P^{32}$ |
|---|---|---|
| Human serum P | 60 | 13 |
| P/50 | 1·2 | 5·4 |
| Human serum B (hypophosphatasia) | 2 | 15·5 |
| B/50 | 0·04 | 5·8 |
| Purified bovine properdin 30 $\mu$/ml. | 0 | 17 |
| Purified human properdin 9 $\mu$/ml. (Wardlaw) | 0 | 16 |
| Properdin (Wardlaw)/10 | 0 | 10 |
| (Wardlaw)/100 | 0 | 7 |
| Rabbit serum | 10 | 8·5 |
| Guinea-pig serum/5 | 2 | 5 |
| Mouse serum/20 | 2 | 18 |

*Relative reactivity of $P^{32}$-LPs from different bacterial strains*

In the course of this work more than 25 separate preparations of $P^{32}$-LP were made from 12 different bacterial strains. The yield of lipopolysaccharide was similar in all cases and successive preparations from the same strain of *Bact. coli* 2206 gave comparable results both as regards yield and degree of hydrolysis by serum. On the other hand, when the samples of $P^{32}$-LP were examined for their reactivity by determining the greatest dilution of pooled rat serum which would cause half maximal hydrolysis there were great differences. On the whole those strains which were most sensitive to killing by rat serum yielded lipopolysaccharides which were hydrolysed by serum at the greatest dilutions (Table IV).

TABLE IV.—*Comparison Between the Hydrolysis of $P^{32}$-LP from Various Bacterial Strains and the Sensitivity of the Parent Strain to Killing by Rat Serum*

| Lipopolysaccharide from strain | Percentage split* $P^{32}$ (I) | Final dilution of rat serum giving approximately 50 per cent splitting | Final dilution of rat serum giving 50 per cent killing of parent strain in 90 min. |
|---|---|---|---|
| *S. paratyphi* B–B IIR | 13–16 | 1/1000 | 1/50 |
| *Bact. coli* Lilly *liq. | 17–22, 15, 17 | 1/400 | 1/50 |
| *Bact. coli* Lilly | 8, 6 | 1/200 | 1/40 |
| *Bact. coli* 2206 | 14 | 1/400 | 1/10 |
| 2206 | 16, 16, 18 | 1/250 | 1/10 |
| 2206 | 16, 19, 20 | 1/100 | — |
| *S. paratyphi* B–$B_I$R | 14, 22, 23 | 1/>100 | 1/50 |
| *Bact. coli* 2206 | 11, 15 | 1/>50 | 1/10 |
| *S. paratyphi* B–$B_{II}$S | 21 | 1/25 | 1/4 |
| $B_I$S | 15, 19, 24 | 1/25 | 1/4 |
| *Bact. coli* 2380 | 10, 13 | 1/20 | N |
| 145 | 11, 14, 16 | 1/10 | >N |

* This batch was prepared from organisms grown in liquid medium.
(I) Each number refers to the results of one experiment.

## DISCUSSION

The finding that phosphate ions can be liberated from lipopolysaccharides by incubation with alkaline phosphatase is not entirely new, since Tal and Goebel (1950) reported that the crude endotoxic complex isolated from *Shig. dysenteriae*

(Flexner) could be attacked by alkaline phosphatase with the liberation of approximately 10 per cent of its phosphorus. On the other hand their material showed an absorption peak at 260 $\mu$m. which disappeared during treatment with alkaline phosphatase, this indicates the presence in their material of nucleic acid and the liberated phosphorus might have come from this.

Present results show that there is an alkaline phosphatase present in the serum of all the animals tested, which appears to have a high degree of specificity for the phosphate-ester bonds of lipopolysaccharide. These same phosphate bonds could be split by other alkaline phosphatases, but there is no correlation between the amount of phenol phosphatase and the amount of lipolysaccharide-phosphatase in any sample of serum, and it seems likely that there are at least two different enzymes involved. This is supported by the finding that serum from a case of hypophosphatasia was as active towards lipopolysaccharide as another sample of serum with a very high phenol phosphatase activity (see Table III).

The suggestion that lipopolysaccharide is a substrate for the serum bactericidal reaction is, at least, not eliminated by these results. Indeed some support for the hypothesis can be derived from the finding that virulent serum-resistant organisms contained lipopolysaccharides which were less rapidly dephosphorylated than those lipopolysaccharides prepared from avirulent serum-sensitive strains. The previous finding that a virulent strain possessed more lipopolysaccharide than an antigenically indistinguishable avirulent one (Rowley, 1956) has not been generally confirmed when more strains have been examined. In fact, we found, in agreement with Westphal and Luderitz (personal communication) that the quantity of LP in different strains of Gram-negative organisms appears to have no relation to the R-S state of the strains or indeed to their virulence for mice.

It is difficult to ascertain whether this serum phosphatase is involved in the complicated events leading to the death of the organism during incubation with fresh serum. Although all the samples of purified properdin which we have examined have possessed lipopolysaccharide-phosphatase activity, evidence from experiments using serum rendered non-bactericidal by removal of properdin suggest that properdin and this enzyme may not be identical, since good lipopolysaccharide splitting was obtained with relatively properdin-free serum. On the other hand it is possible that the serum lipopolysaccharide-phosphatase may be involved in one of the steps leading to bacterial killing, in addition to properdin, magnesium ions and the four components of complement. Until some of these substances are obtained in a pure state it seems unlikely that this interesting question will be satisfactorily resolved.

## SUMMARY

The release of dialysable $P^{32}$ from $P^{32}$-labelled lipopolysaccharides of Gram negative bacteria during incubation with fresh serum is described. The temperature, pH, and partial requirement for divalent cations identify this enzyme as an alkaline phosphatase. Although the rate of reaction with this serum phosphatase is very close to that with phenol phosphatase prepared from calves' intestines, competitive inhibition studies and dilution experiments indicate that the serum enzyme which dephosphorylates these lipopolysaccharides is different from the serum phenol phosphatase in having a more limited substrate specificity.

The rate and extent of hydrolysis was determined for labelled lipopolysaccharides prepared from eight different bacterial strains. There was a correlation

between this and the sensitivity of the parent strain to the bactericidal action of rat serum.

We are indebted to Mr. S. Haskey of this Institute for making the perspex dialysers, to Dr. S. Rowlands of the Physics Department of St. Mary's Hospital Medical School for the alterations to the apparatus used to assay radioactivity, and to Dr. A. C. Wardlaw, of this Institute for supplying and assaying samples of properdin, to Professor L. Pillemer of Western Reserve University, Cleveland, for a generous gift of purified properdin, and to Dr. E. Kroger, Hygiene Institute, Gottingen, Germany, for various rough and smooth pairs of bacteria. The gift of automatic counting equipment from Chas. Pfizer Inc., is gratefully acknowledged and we are indebted to the Medical Research Council for a grant toward the cost of radioactive materials.

## REFERENCES

HARRISON, G. A.—(1947) 'Chemical Methods in Clinical Medicine'. London: (Churchill), p. 365.
MAALOE, O.—(1948) *Acta path. microbiol. scand.*, **25**, 414.
MORTON, R. K.—(1954) *Biochem. J.*, **57**, 595.
ROWLEY, D.—(1954) *Brit. J. exp. Path.*, **35**, 528.—(1956) *Ibid.*, **37**, 223.
*Idem*, HOWARD, J. AND JENKIN, C. R.—(1956) *Lancet*, **i**, 366.
PILLEMER, L., BLUM, L., LEPOW, I. H., ROSS, O. A., TODD, E. N. AND WARDLAW, A. C. —(1954) *Science*, **120**, 279.
TAL, C. AND GOEBEL, W. F.—(1950) *J. exp. Med.*, **92**, 25.
WESTPHAL, O., LUDERITZ, O. AND BISTER, F.—(1952) *Z. Naturw.*, **76**, 148.

# SPECIES AND SEX DIFFERENCES IN FATTY ACID RELEASE BY TISSUES INCUBATED WITH LIPAEMIC HUMAN SERUM

T. ZEMPLÉNYI AND D. GRAFNETTER

*From the Institute for Cardiovascular Research, Prague, Czechoslovakia*

Received for publication December 2, 1957

It has been shown (Zemplényi and Grafnetter, 1957) that incubation of rat tissue *brei* with lipaemic human serum is a useful technique for demonstrating the lipolytic (lipoproteinolytic) activity of various tissues, and the increase in this activity (up to 150 per cent) produced by heparin. These results are in agreement with Korn's findings with lipoprotein lipase (Korn 1954, 1955 ; Korn and Quigley, 1955). It has also been shown that fasting produces a marked rise in lipolytic activity of the rat myocardium (Zemplényi and Grafnetter, 1957).

One of the most characteristic features of human atherosclerosis is its lower incidence in females up to the menopause (Katz and Stamler, 1953). Some factors which bear a relationship to the pathogenesis of atherosclerosis show clear sexual differences, *e.g.*, Barr's data on the blood lipid spectrum of males and females (Barr, 1953). A striking feature of experimental atherosclerosis is the different susceptibility of various species. Rabbits are extremely susceptible, as has been repeatedly confirmed from the time of Anitchkow's first experiments (Anitschkow, 1913), but only a few authors have been able to produce atherosclerotic changes in rats (Malinow, Hojman and Pellegrino, 1954 ; Hartroft, Ridout, Sellers and Best, 1949 ; Bragdon and Boyle, 1952). We have been able to produce only occasional lipoid infiltration of the aorta in rats on a cholesterol diet with thiouracil administration (Felt, Lojda, Zemplényi and Fodor, 1956), which cannot be considered to be identical with atherosclerotic changes (Page and Brown, 1952).

The following questions are considered in the present communication :

(*a*) Are there species differences in tissue lipolytic activity in species susceptible to experimental atherosclerosis, using the rat as a standard of comparison ?

(*b*) Is there a sex-linked difference in tissue lipolytic activity ?

## METHODS

The following animals were used : white Wistar rats, body weight 150–250 g., white Leghorn cocks, 4 months old, rabbits, guinea-pigs and golden hamsters.

The rats were fed on a Larsen diet, the other species on the ordinary laboratory diet usually given these species.

All animals were fasted 3–6 hr. before each experiment.

The rabbits were killed by a blow on the cervical vertebrae and haemorrhage from the cervical vessels. The other species were sacrificed by decapitation. Immediately after killing the required organs were removed, cut up, and washed and stored in physiological saline at 0° for some minutes. On removal from the saline the organ segments were dried on filter paper and rapidly weighed on a torsion balance. In all experiments, except those using aorta, tissue samples were of the order of 120 mg. Aorta samples—carefully cleaned of periaortic tissue—weighed less (Table II), and in experiments with hamsters, it was necessary to combine two aorta samples in a single incubation flask in order to have a sufficient quantity

of tissue. Weighed samples of tissue were minced into small pieces on a glass plate, and these pieces were then further minced by rapid vertical strokes of a razor blade. In this manner heart, liver, lung and kidney were used to prepare a suitable fairly homogeneous tissue *brei*. Aorta and adipose tissue were prepared only by cutting into small pieces with scissors strokes. Preparation of the tissue *brei* lasted about 1 min. in all.

Lipolytic activity of individual tissues was estimated in terms of the amount of unesterified fatty acids liberated in m.equiv./l./g. of tissue. Substrate was lipaemic human (male) serum separated from blood withdrawn 2½ to 3 hr. after a meal of 300 ml. of whipped cream. Serum separated in this manner was diluted 1 : 2 with Sörensen phosphate buffer, pH 7·38 ($6{\cdot}6 \times 10^{-2}$ M). Incubation was carried out in a water bath at 37° with constant shaking of the vessels, for an interval of 150 min. Each incubation vessel contained 3 ml. of medium, to which was added a weighed amount of tissue *brei*. The vessels were then immediately placed in the bath and 7 min. later 1 ml. of medium was pipetted out for estimation of initial levels of unesterified fatty acids. A second 1 ml. sample was pipetted off at 150 min.

Unesterified fatty acids were estimated by the method of Dole (1956). One ml. of the sample was added to 5 ml. of the extraction mixture (isopropyl alcohol, heptane and N$H_2SO_4$ in a ratio of 40 : 10 : 1) in a glass-stoppered cylinder, and well mixed. After 10 min. 3 ml. of heptane and 2 ml. of distilled water were added, and the mixture shaken once again. After phase separation, 3 ml. from the upper layer were pipetted off into titration tubes, and titrated in the presence of 1 ml. of indicator solution (Nile Blue in 90 per cent alcohol) with 0·018 N NaOH added from a microburette. The NaOH standard was calibrated by extraction and titration of a standard solution of sodium oleate (Merck, A. R.). The calibration curve from 0 to 8 m. equiv./l. lay on a straight line passing through the origin.

The concentration of unesterified fatty acids was expressed in m.equiv./l. The difference in concentration of unesterified fatty acids in the incubation medium at 150 and 7 min. constitutes a measure of the degree of lipolysis Since the absolute amount of tissue in individual samples in these experiments fell within fairly narrow limits, the results were calculated on the basis of 1 g. of tissue, *i.e.*:

$$\frac{\text{m.equiv. unesterified fatty acids/l.} \times 1000 \text{ mg.}}{\text{Tissue weight (mg.)}}$$

The substrate was, of course, from a different donor on each experimental day. In order to compare experiments carried out on different days, results were calculated in two experimental series (Figs. 1 and 3) as percentage differences, using rat tissue values as 100 per cent for comparison with other species. In the other series (Tables I and II and Fig. 2, 4, 5, 6), absolute results, *i.e.*, m.equiv./l./g. tissue, have been presented, since these results were obtained during the course of one day's experimental observation.

## RESULTS

### *Comparison of lipolysis (freed unesterified fatty acids in m.equiv./l./g. tissue) by the organs of male rats and rabbits*

The groups consisted of 6 animals of each species. Fig. 1 shows the marked differences in tissue activity, rabbit heart having a greater activity than rat ($P < 0{\cdot}01$). The same is true for lung ($P < 0{\cdot}05$). Liver and adipose tissue show a reversed ratio of activity, the higher lipolytic activity being in rat liver and perirenal fat ($P < 0{\cdot}001$).

Fig. 2 shows an illustrative example of lipolytic activation in rat and rabbit tissue on incubation in the presence of heparin ($1{\cdot}6 \times 10^{-5}$ M).

### *Comparison of lipolysis by tissues of female rats and rabbits*

The groups consisted of 6 animals of each species. Fig. 3 shows striking differences as opposed to the results obtained with males of the same species. Rabbit heart activity was markedly lower than rat ($P < 0{\cdot}001$) while rabbit

adipose tissue was more active than rat ($P < 0{\cdot}05$). The comparative relationships are about the same as with males for the other tissues (lung $P < 0{\cdot}05$ and liver $P < 0{\cdot}001$). The results with kidney cannot be evaluated due to the small number of experimental results.

*Comparison of lipolysis by tissues of cocks and male rats*

Tissue lipolytic activity was compared from the organs of 4 cocks and 4 male rats. Fig. 4 and Table I show, despite the small number of animals, that cock heart has a higher lipolytic activity, and cock liver a lower activity, than the

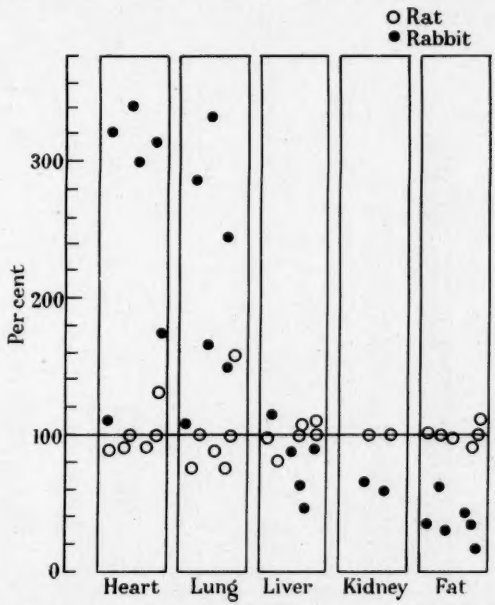



FIG. 1.—Lipolysis by tissues from male rats and rabbits. 1·5 ml. of male lipaemic human serum was diluted with phosphate buffer, pH 7·38 ($6{\cdot}6 \times 10^{-2}$ M) and incubated with a weighed amount of tissue *brei* at 37° under constant shaking in the bath. Unesterified fatty acids (NEFA) measured at 7 and 150 min. Average activity of NEFA/l./g. tissue is arbitrarily set for the rat at 100 per cent.

corresponding rat tissues, while the lung results overlap for the two species. It was not possible to compare adipose tissue, for we could not find fat in the cock which would correspond to perirenal or lumbar fat in the rat. The results here show the same comparative relationship as those in the groups of rabbit and rat males.

*Comparison of lipolysis by tissues from hamsters and rats*

Fig. 5 and Table I show the ratio of organ activity in 5 golden hamsters and 5 rats (males). The picture is similar to that already presented above: a high cardiac ($P < 0{\cdot}001$) and lung ($P < 0{\cdot}01$) activity in comparison with rat organs.

*Comparison of lipolysis by tissues of male guinea-pigs and rats*

The results obtained from 4 guinea-pigs and 4 rats are shown in Table I. Once again, cardiac activity is higher in the guinea-pig ($P < 0.02$). We were able to carry out only 2 experiments with lung from these two species, and no differences were demonstrated.

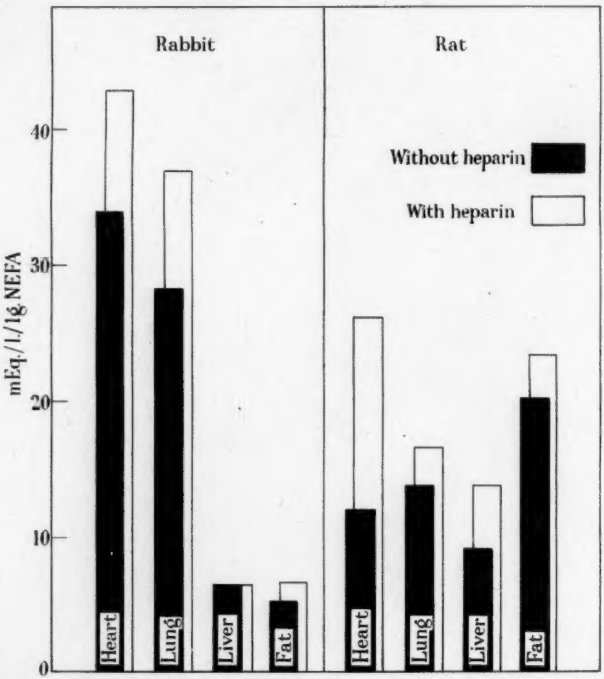



FIG. 2.—Activation of lipolysis in rat and rabbit tissues by heparin. Experimental conditions as in Fig. 1. $1.6 \times 10^{-5}$ M heparin added to the experimental vessels. Results in m.equiv.NEFA/l./g. tissue.

*Lipolytic activity in the aorta*

Results from incubation of the thoracic aorta of animals from the above experimental series are presented in Table II. Although the method of chemical analysis is very precise, the experimental approach is such that only differences greater than 0·13 m.equiv. of unesterified fatty acids can be taken as significant, while differences of less than 0·07 m.equiv. can be ignored. The table shows that rat aorta has a very high activity in comparison with rabbit aorta, the latter activity being practically *nil* in most cases. The same comparison is true for guinea-pig and rat. The levels from hamsters and cocks cannot be evaluated, since they are too close to the limit of error of the method, although there is some tendency for a higher activity in the rat.

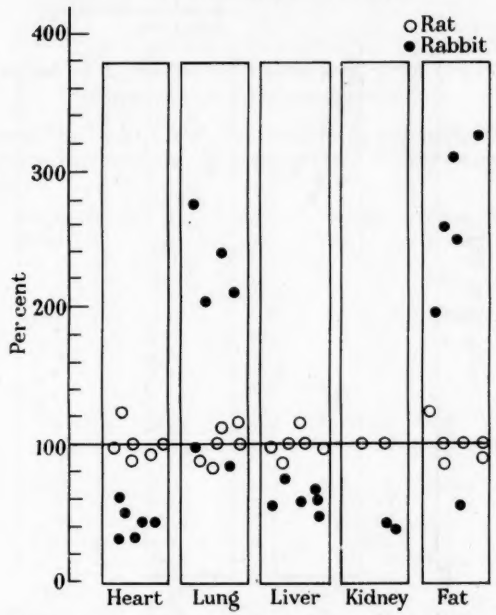



FIG. 3.—Lipolysis by tissues from female rats and rabbits. Conditions as in Fig. 1.

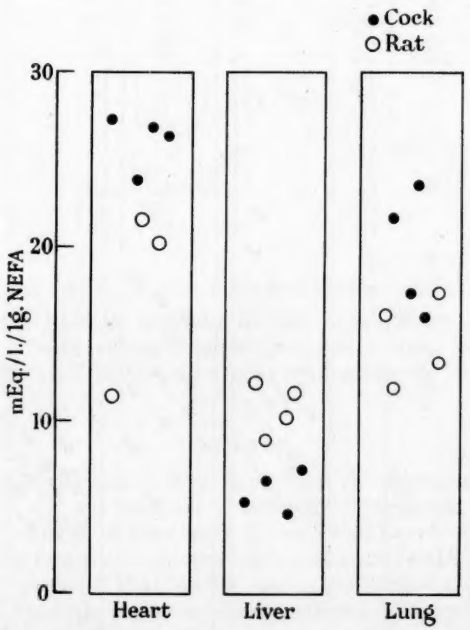



FIG. 4.—Lipolysis by tissues from cocks and male rats. Conditions as in Fig. 1. Results in m. equiv.NEFA/l./g. tissue.

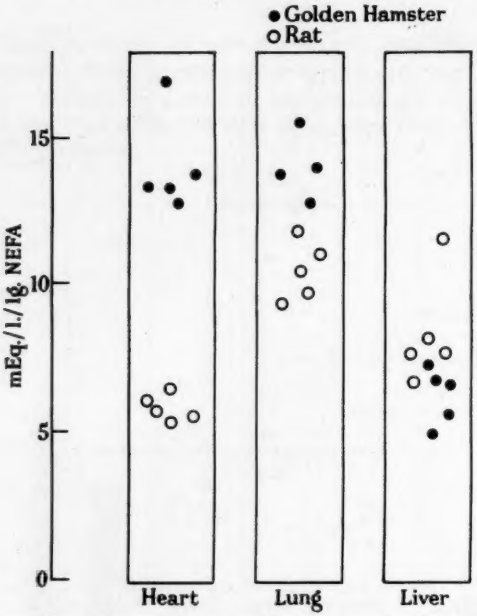



FIG. 5.—Lipolysis by tissues from male rats and hamsters. Conditions as in Fig. 1 and 4.

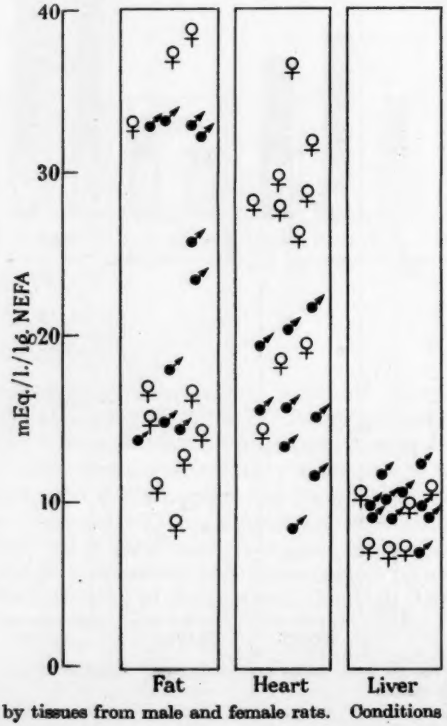



FIG. 6.—Lipolysis by tissues from male and female rats. Conditions as in Fig. 1 and 4.

TABLE I.—*Comparison of Lipolysis by Tissue* brei *from Organs of Rats, Cocks, Guinea-pigs and Golden Hamsters*

Conditions as in Fig. 1 and 4. " A " is the difference in m.equiv./l. between 7 and 150 min. of incubation. " B " represents " A " values calculated per gram of tissue.

| Date | Animal | | Tissue | Weight | A | B | Animal | | Tissue | Weight | A | B |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17.vi | Cock | I | Heart | 121 | 3·3 | 27·27 | Rat | I | Heart | 120 | 1·36 | 11·33 |
| | | II | „ | 117 | 3·13 | 26·75 | | II | „ | 119 | 2·58 | 21·68 |
| | | III | „ | 117 | 3·09 | 26·41 | | III | „ | 123 | 2·48 | 20·16 |
| | | IV | „ | 123 | 2·92 | 23·74 | | IV | „ | — | — | — |
| | | I | Lung | 122 | 2·65 | 21·72 | | I | Lung | 115 | 1·36 | 11·83 |
| | | II | „ | 113 | 2·65 | 23·45 | | II | „ | 123 | 2·11 | 17·15 |
| | | III | „ | 123 | 2·11 | 17·15 | | III | „ | 119 | 1·90 | 15·97 |
| | | IV | „ | 118 | 1·87 | 15·85 | | IV | „ | 118 | 1·56 | 13·22 |
| | | I | Liver | 123 | 0·64 | 5·20 | | I | Liver | 118 | 1·36 | 11·52 |
| | | II | „ | 118 | 0·75 | 6·35 | | II | „ | 119 | 1·05 | 8·82 |
| | | III | „ | 120 | 0·54 | 4·50 | | III | „ | 118 | 1·19 | 10·08 |
| | | IV | „ | 120 | 0·88 | 7·10 | | IV | „ | 115 | 1·39 | 12·09 |
| 25.vi | G.-pig | I | Heart | 119 | 2·45 | 20·59 | Rat | V | Heart | 120 | 1·70 | 14·17 |
| | | II | „ | 117 | 3·06 | 26·15 | | VI | „ | 120 | 1·63 | 13·58 |
| | | III | „ | 121 | 3·64 | 30·10 | | VII | „ | 119 | 1·49 | 12·52 |
| | | IV | „ | 123 | 4·32 | 35·12 | | VIII | „ | 121 | 1·60 | 13·22 |
| | | I | Lung | 118 | 1·02 | 8·64 | | V | Lung | 120 | 1·70 | 14·17 |
| | | II | „ | 119 | 1·97 | 16·55 | | VI | „ | 123 | 1·43 | 11·63 |
| 2.vii | G. ham. | I | Heart | 119 | 1·60 | 13·42 | Rat | IX | Heart | 119 | 0·73 | 6·13 |
| | | II | „ | 119 | 1·53 | 12·84 | | X | „ | 122 | 0·66 | 5·40 |
| | | III | „ | 120 | 1·67 | 13·89 | | XI | „ | 118 | 0·66 | 5·59 |
| | | IV | „ | 120 | 1·60 | 13·40 | | XII | „ | 122 | 0·80 | 6·54 |
| | | V | „ | 120 | 2·05 | 17·07 | | XIII | „ | 119 | 0·69 | 5·83 |
| | G. ham. | I | Lung | 120 | 1·88 | 15·63 | Rat | IX | Lung | 122 | 1·15 | 9·49 |
| | | II | „ | 118 | 1·67 | 14·12 | | X | „ | 120 | 1·18 | 9·84 |
| | | III | „ | 118 | 1·63 | 13·83 | | XI | „ | 119 | 1·42 | 11·96 |
| | | IV | „ | 119 | 1·53 | 12·84 | | XII | „ | 118 | 1·25 | 10·59 |
| | | V | „ | — | — | — | | XIII | „ | 118 | 1·25 | 10·59 |
| | | I | Liver | 117 | 0·59 | 5·04 | | IX | Liver | 122 | 0·83 | 6·83 |
| | | II | „ | 121 | 0·70 | 5·74 | | X | „ | 121 | 1·01 | 8·32 |
| | | III | „ | 122 | 0·83 | 6·83 | | XI | „ | 121 | 1·42 | 11·76 |
| | | IV | „ | 119 | 0·80 | 6·71 | | XII | „ | 121 | 0·94 | 7·75 |
| | | V | „ | 122 | 0·90 | 7·40 | | XIII | „ | 121 | 0·94 | 7·75 |

*Comparison of lipolysis by tissues from male and female rats*

Fig. 6 shows that lipolytic activity in the myocardium is much higher in females ($P < 0{\cdot}001$), while values from adipose tissue show scatter and there is no significant difference. This last statement applies also to liver activity.

## DISCUSSION

Few questions in lipid metabolism have been so extensively studied as the " clearing reaction ", *i.e.*, the decrease in turbidity of lipaemic serum after intravenous injection of heparin. Korn's work (1954, 1955) has shown a close relationship between clearing factor and lipoprotein lipase contained in myocardium and fat tissue. It is very probable that the enzyme system whose activity is estimated on incubation with lipaemic serum is closely related to lipoprotein lipase (Zem-

TABLE II.—*Comparison of Lipolytic Activity of Aortae.*

Conditions as in Fig. 1. "A" is the difference in NEFA in m.equiv./l. between 7 and 150 min. "B" represents "A" values calculated per gram of tissue. *Negligible values.

| Date | Animal | | Aorta (mg.) | A | B | Animal | | Aorta (mg.) | A | B |
|---|---|---|---|---|---|---|---|---|---|---|
| 21.v | Rabbit | I | 58 | 0·07* | — | Rat | XIV | 42 | 0·92 | 21·9 |
| | | II | 61 | 0·03* | — | | XV | 36 | 0·78 | 21·66 |
| 25.v | | III | 44 | 0·14 | 3·19 | | XVI | 36 | 0·75 | 20·80 |
| | | IV | 42 | 0·12 | 2·91 | | XVII | 44 | 0·41 | 9·32 |
| 29.v | | V | 45 | 0·07* | — | | XVIII | 41 | 0·54 | 13·2 |
| | | VI | 46 | 0·07* | — | | XIX | 29 | 0·27 | 9·30 |
| | | VII | 47 | 0·03* | — | | XX | 47 | 0·41 | 8·70 |
| | | VIII | 46 | 0·07* | — | | XXI | 34 | 0·17 | 5·00 |
| 3.vi | | IX | 40 | 0·03* | — | | XXII | 40 | 0·27 | 6·75 |
| | | X | 43 | 0·03* | — | | XXIII | 36 | 0·24 | 6·67 |
| | | XI | 52 | 0·10 | 1·92 | | XXIV | 36 | 0·24 | 6·67 |
| | | XII | 44 | 0·03* | — | | XXV | 30 | 0·24 | 8·00 |
| 16.vi | | XIII | 106 | 0·18 | 1·69 | | XXVI | 70 | 1·41 | 20·15 |
| 17.vi | Cock | I | 42 | 0·20 | 4·76 | Rat | I | 21 | 0·17 | 8·10 |
| | | II | 39 | 0·13 | 3·33 | | II | 24 | 0·20 | 8·33 |
| | | III | 47 | 0·13 | 2·76 | | III | 32 | 0·30 | 9·37 |
| | | IV | 45 | 0·13 | 2·89 | | IV | 24 | 0·13 | 5·42 |
| 25.vi | G.-pig | I | 34 | 0·20 | 5·88 | Rat | V | 37 | 0·64 | 17·30 |
| | | II | 30 | 0·00 | 0·00 | | VI | 30 | 0·30 | 10·00 |
| | | III | 28 | 0·17 | 6·07 | | VII | 32 | 0·44 | 13·75 |
| | | IV | 27 | 0·03* | — | | VIII | 29 | 0·30 | 10·34 |
| 2.vii | G. ham. | I–II | 12 | 0·14 | 11·80 | Rat | IX | 12 | 0·17 | 14·17 |
| | | III–IV | 14 | 0·14 | 10·00 | | X | 20 | 0·17 | 8·50 |
| | | V–VI | 22 | 0·10 | 5·55 | | XI | 14 | 0·28 | 20·00 |
| | | | | | | | XII | 22 | 0·35 | 15·90 |
| | | | | | | | XIII | 20 | 0·14 | 7·00 |

plényi and Grafnetter, 1957). It would appear that under physiological conditions, clearing factor only exceptionally appears in the vascular compartment, and that physiological "clearing" is a tissue rather than a blood phenomenon (Bergström and Borgström, 1956).

Previous investigators, using simple triglycerides as substrate have noted only slight lipolytic activity in adipose tissue (see Korn and Quigley, 1955). Renold and Marble (1950) found maximal tissue lipolytic activity in liver and pancreas rather than lung and adipose tissue; however they used Tween 20 as substrate and these results cannot be clearly equated with lipoproteinolytic activity.

The results presented here show that tissues from males of various species do not show the same level of activity on incubation with lipaemic human (male) serum. The most constant finding is that of a low lipolytic activity in the myocardium of rats, in comparison with the same organ from the rabbit, cock, guinea-pig and golden hamster. Just as interesting is the opposite ratio of activities, *i.e.*, higher activity in rat tissues, as opposed to rabbit, cock, hamster (in liver) and as opposed to rabbit (in adipose tissue).

The above species have a similar interrelationship in susceptibility to experimental cholesterol atherosclerosis, even if there is no unanimity as to the susceptibility of the hamster (Goldman and Pollak, 1949). The finding of basically identical

comparative relationships of tissue activities suggests some relation to the cause of differential species susceptibility and ultimately to the pathogenic basis of atherosclerosis. Additional knowledge is required of the physiological role of tissue lipolysis in fat metabolism and the relative importance of myocardium, lung, and particularly adipose tissue and liver, in this process.

A further surprising finding was the high level of lipolytic activity in the rat aorta in comparison with rabbit, and more or less in comparison with aorta from all the other species, as far as reliable comparison was available. A possible relation of this to the pathogenesis of atherosclerosis may be that in a tissue with a low lipolytic activity conditions exist for the accumulation of triglycerides and cholesterol, the latter being partly maintained in solution by triglycerides (Albrink, Man and Peters, 1955 ; Friedman and Byers, 1955 ; Hirsch and Nailor, 1956).

This question, however, has another aspect : many authors concerned with the problem of species differences in susceptibility to atherosclerosis have found interesting differences in the properties of vessel walls (Page and Brown, 1952 ; Schlichter and Harris, 1949), or in the biochemical properties of blood and in metabolism in general (Barr, 1953 ; Friedman and Byers, 1954 ; Lewis, Green and Page, 1952 ; Pilgeram and Greenberg, 1954). If the tissue lipolytic enzyme system is identical with the clearing factor, which is in all probability one of the regulating factors in lipid metabolism and of the lipid spectrum in the blood (Anfinsen, 1954), the results on aorta activity would, in this case, indicate a pathogenic factor, both vascular and regulatory.

Cairns and Constantinides (1955) have shown a relationship between clearing factor activity and hormonal factors. Our experiments clearly show a relationship between tissue lipolytic activity and sex.

If we assume that tissue lipolysis has metabolic significance the finding of a sexual difference is not surprising, since lipid metabolism *per se* shows sex-linked differences (Deuel, 1956).

## SUMMARY

Incubation of a tissue *brei* with lipaemic human serum and estimation of freed unesterified fatty acids enables the lipolytic (lipoproteinolytic) activity of tissues to be estimated.

Organs from the rat, and from other species more susceptible to experimental atherosclerosis (rabbit, cock, guinea-pig, hamster) were compared for lipolytic activity using human lipaemic serum (male) as substrate. Heart and lung from male rats have a lower activity, liver and fat tissue a higher activity, than rabbit males. The other species show the same approximate relationship to the rat, although they were not examined in the same detail. In females, rats and rabbits, the ratio of activities is the reverse in myocardium and adipose tissue.

Rat aorta has a marked lipolytic activity, rabbit aorta practically none. Lipolysis by guinea-pig aorta, in relation to the rat, is low.

Female rat myocardium has a higher lipolytic activity than male myocardium.

The possible relation of the above to the pathogenesis of atherosclerosis is discussed.

The authors would like to thank Mrs. J. Hájková for technical assistance, and M. Bartoníček for statistical calculations.

## REFERENCES


ALBRINK, M. J., MAN, E. B. AND PETERS, J. F.—(1955) *J. clin. Invest.*, **34**, 147.
ANFINSEN, CH. B.—(1954) *In* 'Fat Metabolism'. Baltimore (The Johns Hopkins Pr.).
ANITSCHKOW, N. N.—(1913) *Beitz. z. Allg. Anat. u. Path.*, **56**, 379.
BARR, D. P.—(1953) *Circulation*, **8**, 641.
BERGSTRÖM, S. AND BORGSTRÖM, B.—(1956) *Annu. Rev. Biochem.*, **25**, 177.
BRAGDON, J. H. AND BOYLE, E.—(1952) *Amer. J. Path.*, **38**, 527.
CAIRNS, A. AND CONSTANTINIDES, P.—(1955) *Canad. J. Biochem.*, **33**, 530.
DEUEL, H. J., Jr.—(1956) *Int. Z. Vitaminforsch.*, **26**, 352.
DOLE, V. P.—(1956) *J. clin. Invest.*, **35**, 150.
FELT, V., LOJDA, Z., ZEMPLÉNYI, T. AND FODOR, J.—(1956) *Proc. Congr. Czechosl. Physiol. Soc.*
FRIEDMAN, M. AND BYERS, S. O.—(1954) *Amer. J. Physiol.*, **179**, 201.—(1955) *Proc. Soc. exp. Biol., N.Y.*, **90**, 496.
GOLDMAN, J. AND POLLAK, O. J.—(1949) *Amer. Heart J.*, **38**, 474.
HARTROFT, W. S., RIDOUT, J. H., SELLERS, E. A. AND BEST, C. H.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **81**, 384.
HIRSCH, E. F. AND NAILOR, R.—(1956) *Arch. Path.*, **61**, 469.
KATZ, L. N. AND STAMLER, J.—(1953) In 'Experimental Atherosclerosis'. Springfield, Illinois.
KORN, E. D.—(1954) *Science*, **120**, 399.—(1955) *J. biol. Chem.*, **215**, 1.
*Idem* AND QUIGLEY, TH. W. Jr.—(1955) *Biochim. biophys. Acta*, **18**, 143.
LEWIS, L. A., GREEN, A. A. AND PAGE, I. H.—(1952) *Amer. J. Physiol.*, **171**, 391.
MALINOW, M. R., HOJMAN, D. AND PELLEGRINO, A.—(1954) *Acta cardiol., Brux.*, **9**, 480.
PAGE, I. H. AND BROWN, H. B.—(1952) *Circulation*, **6**, 681.
PILGERAM, L. O. AND GREENBERG, D. M.—(1954) *Science*, **120**, 760.
RENOLD, A. E. AND MARBLE, A.—(1950) *J. biol. Chem.*, **185**, 367.
SCHLICHTER, J. G. AND HARRIS, R.—(1949) *Amer. J. med. Sci.*, **218**, 610.
ZEMPLÉNYI, T. AND GRAFNETTER, D.—(1958) *Science*, in press.

# SURVEY OF PAPERS.

STEVENS and HELEN C. PIETRYK, developing an observation made by Billingham, Brent and Medawar on the acquisition of immunological tolerance by mice and chickens during the embryonic stage, have studied the immunological response of chickens grown from fertile eggs which had been injected in the yolk or extra-allantoically with soluble human serum albumin whilst immunological reactions were acquired the tolerance was not complete and the results have been analysed statistically by J. L. Ciminera. Attempts to correlate time of inoculation, sex, age and body weight of the experimental animals have been made. The acquisition of immunological tolerance was more successful when the antigen was injected extra-allantoically (p. 1).

CINADER and PEARCE have studied the acquisition of tolerance by the rabbit to various azo-proteins and in particular whether tolerance is directed towards the protein or azo moiety. Tolerance to azo human albumin was conferred by a number of injections beginning within 12 hours of birth and the survival of human albumin in these animals was increased. One animal tolerant to human albumin responded to the azo-protein with antibody directed primarily against the modified determinants. Animals injected at birth with azo rabbit serum or azo ribonuclease gave the same antibody titres to azo human albumin as the controls but in those pre-injected with azo ribonuclease the antibody was slightly less well-adapted to the modified determinants (p. 8).

BLISS, STEWART and FULLER show that inbred dogs of standard pure breeds react to a first intradermal injection of non-autologous plasma in a similar manner to mongrels. This effect persists even in very closely related animals, showing that the degree of individual specificity is very high. The inoculation of plasma from mongrel puppies into adult animals often failed to produce typical responses (p. 30).

ACHESON and MACINTYRE subjected groups of three-week-old rats to pneumococcal septicaemia (treated with penicillin) and intermittent starvation. The surviving animals were retarded in length, weight and skeletal maturity as compared with the controls. The post-infective animals had caught up with their controls by the age of 16 weeks but the post-starvation animals showed residual stunting of their growth (p. 37).

STAVITSKY shows that the transfer of morphologically intact cells from immunised to normal rabbits gives rise in the recipient to amounts of antibody greatly in excess of the cellular protein or globulin transferred. This appears to result from new protein synthesis from free amino acids, since, provided breakdown does not occur, labelled TCA insoluble potential precursors were not incorporated into antibody while $S^{35}$ labelled methioline was (p. 46).

GOLBERG and SMITH induced gross iron overloading in rats by the administration of intramuscular iron to a total of 1·65 g. Fe/kg. body weight. The sequelae resembled many of the pathological and biochemical changes previously observed in rats deficient in Vitamin E. Some of these changes are accentuated by cod liver oil in the diet while Vitamin E exercises a protective action. Three types of ceroid-like polymeric pigments are found in the tissues and their mode of formation is described (p. 59).

DONNA CHAPRONIERE, DONE and ANDREWES obtained growth of Talfan virus with cytopathic effect in tissue cultures of piglet kidney and demonstrated close similarities and cross-neutralization between it and the virus of Teschen disease (p. 74).

BURKE and ISAACS have found that maximal yields of interferon from the chick chorion were obtained with influenza virus treated for a short time with ultra-violet irradiation.

Larger amounts of heat-inactivaed virus were required; incomplete virus produced good results. Slight damage to virus nucleic acid is thought to be necessary to initiate interferon synthesis (p. 78).

RUEBNER and JANET MCLAREN show that the high ammonia levels normally present in the mesenteric veins of rabbits are greatly reduced in animals receiving oxytetracycline. This effect does not occur in animals treated with neomycin. They produce evidence that oxytetracycline exerts this effect by greatly reducing organisms of the *Bacteroides* group. They suggest that treatment of patients with cirrhosis of the liver with suitable antibiotics would be beneficial since ammonia is toxic to sufferers from this condition (p. 85).

ROWLEY, ALI and JENKIN describe the preparation of $p^{32}$ labelled bacterial lipopolysaccharide and compare dephosphorylation of this by fresh serum and calf intestine alkaline phosphates. The serum enzyme involved has less effect on bacterial strains resistant to the bactericidal effect of serum and appear to be distinct from phenol phosphatase (p. 90).

ZEMPLÉNYI and GRAFNETTER, using rats, Leghorn cocks, rabbits, guinea-pigs and golden hamsters, assess species and sex differences in tissue lipolytic activity by incubation of a tissue *brei* with lipaemic human serum and estimating the freed unesterified fatty acids. Heart and lung from male rats had a lower activity, and liver and fat a higher activity than male rabbits. In female rats and rabbits the myocardial and adipose tissue activity was reversed. These findings are discussed in relation to the problem of atherosclerosis (p. 99).